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(54) Title: 5' NUCLEASES DERIVED FROM THERMOSTABLE DNA POLYMERASE

(57) Abstract

A means for cleaving a nucleic acid cleavage structure in a site-specific manner is disclosed. A cleaving enzyme having 5' nuclease activity without interfering nucleic acid synthetic ability is employed as the basis of a novel method of detection of specific nucleic acid sequences.

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5' NUCLEASES DERIVED FROM THERMOSTABLE DNA POLYMERASE

This is a Continuation-In-Part Application of Application Serial No. 08/073,384, filed June 4, 1993, which is a Continuation-In-Part Application of Application Serial No. 07/986,330, filed December 12, 1992.

FIELD OF THE INVENTION

The present invention relates to means for cleaving a nucleic acid cleavage structure in a site-specific manner. In particular, the present invention relates to a cleaving enzyme having 5' nuclease activity without interfering nucleic acid synthetic ability.

BACKGROUND OF THE INVENTION

The detection of specific nucleic acid sequences has been utilized to diagnose the presence of viral or bacterial nucleic acid sequences indicative of an infection, the presence of variants or alleles of mammalian genes associated with disease and the identification of the source of nucleic acids found in forensic samples and in paternity determinations.

The detection of specific nucleic acid sequences has been achieved typically by hybridization. Hybridization methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be detected). The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, *Proc. Natl. Acad. Sci. USA* 46:453 (1960) and Doty *et al.*, *Proc. Natl. Acad. Sci. USA* 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

Initial hybridization studies, such as those performed by Hayashi *et al.*, *Proc. Natl. Acad. Sci. USA* 50:664 (1963), were formed in solution. Further development led to the immobilization of the target DNA or RNA on solid supports. With the discovery of specific restriction endonucleases by Smith and Wilcox, *J. Mol. Biol.* 51:379 (1970), it became possible to isolate discrete

fragments of DNA. Utilization of immobilization techniques, such as those described by Southern, *J. Mol. Biol.* 98:503 (1975), in combination with restriction enzymes, has allowed for the identification by hybridization of single copy genes among a mass of fractionated, genomic DNA.

In spite of the progress made in hybridization methodology, a number of problems have prevented the wide scale use of hybridization as a tool in human diagnostics. Among the more formidable problems are: 1) the inefficiency of hybridization; 2) the low concentration of specific target sequences in a mixture of genomic DNA; and 3) the hybridization of only partially complementary probes and targets.

1. Inefficient Hybridization

It is experimentally observed that only a fraction of the possible number of probe-target complexes are formed in a hybridization reaction. This is particularly true with short oligonucleotide probes (less than 100 bases in length). There are three fundamental causes: a) hybridization cannot occur because of secondary and tertiary structure interactions; b) strands of DNA containing the target sequence have rehybridized (reannealed) to their complementary strand; and c) some target molecules are prevented from hybridization when they are used in hybridization formats that immobilize the target nucleic acids to a solid surface.

Even where the sequence of a probe is completely complementary to the sequence of the target, *i.e.*, the target's primary structure, the target sequence must be made accessible to the probe via rearrangements of higher-order structure. These higher-order structural rearrangements may concern either the secondary structure or tertiary structure of the molecule. Secondary structure is determined by intramolecular bonding. In the case of DNA or RNA targets this consists of hybridization within a single, continuous strand of bases (as opposed to hybridization between two different strands). Depending on the extent and position of intramolecular bonding, the probe can be displaced from the target sequence preventing hybridization.

Solution hybridization of oligonucleotide probes to denatured double-stranded DNA is further complicated by the fact that the longer complementary

target strands can renature or reanneal. Again, hybridized probe is displaced by this process. This results in a low yield of hybridization (low "coverage") relative to the starting concentrations of probe and target.

The immobilization of target nucleic acids to solid surfaces such as nylon or nitrocellulose is a common practice in molecular biology. Immobilization formats eliminate the reassociation problem that can occur between complementary strands of target molecules, but not the problems associated with secondary structure effects. However, these mixed phase formats (*i.e.*, Southern hybridization or dot blot hybridization) require time consuming fixation procedures. The hybridization reaction itself is kinetically much slower than a solution phase hybridization reaction. Together, the fixation and hybridization procedures require a minimum of several hours to several days to perform. Additionally, the standard immobilization procedures are often inefficient and result in the attachment of many of the target molecules to multiple portions on the solid surface, rendering them incapable of subsequent hybridization to probe molecules. Overall, these combined effects result in just a few percent of the initial target molecules being bound by probes in a hybridization reaction.

2. Low Target Sequence Concentration

In laboratory experiments, purified probes and targets are used. The concentrations of these probes and targets, moreover, can be adjusted according to the sensitivity required. By contrast, the goal in the application of hybridization to medical diagnostics is the detection of a target sequence from a mixture of genomic DNA. Usually the DNA fragment containing the target sequence is in relatively low abundance in genomic DNA. This presents great technical difficulties; most conventional methods that use oligonucleotide probes lack the sensitivity necessary to detect hybridization at such low levels.

One attempt at a solution to the target sequence concentration problem is the amplification of the detection signal. Most often this entails placing one or more labels on an oligonucleotide probe. In the case of non-radioactive labels, even the highest affinity reagents have been found to be unsuitable for the detection of single copy genes in genomic DNA with oligonucleotide probes. *See* Wallace

et al., *Biochimie* 67:755 (1985). In the case of radioactive oligonucleotide probes, only extremely high specific activities are found to show satisfactory results. *See* Studencki and Wallace, *DNA* 3:1 (1984) and Studencki *et al.*, *Human Genetics* 37:42 (1985).

Polymerase chain reaction (PCR) technology provides an alternate approach to the problems of low target sequence concentration. PCR can be used to directly increase the concentration of the target prior to hybridization. In U.S. Patents Nos. 4,683,195 and 4,683,202, Mullis *et al.* describe a method for increasing the concentration of a segment of target sequence in a mixture of genomic DNA without cloning or purification.

This process for amplifying the target sequence consists of introducing a molar excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence. The two primers are complementary to their respective strands of the double-stranded sequence. The mixture is denatured and then allowed to hybridize. Following hybridization, the primers are extended with polymerase so as to form complementary strands. The steps of denaturation, hybridization, and polymerase extension can be repeated as often as needed to obtain relatively high concentration of a segment of the desired target sequence. The length of the segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and, therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to by the inventors as the "Polymerase Chain Reaction" (or PCR). Because the desired segment of the target sequence become the dominant sequences (in terms of concentration) in the mixture, they are said to be "PCR-amplified."

However the PCR process is susceptible to the production of non-target fragments during the amplification process. Spurious extension of primers at partially complementary regions occurs during PCR reactions. Factors influencing the specificity of the amplification process include: a) the concentration of the target sequence in the DNA to be analyzed; b) the concentration of the Mg^{++}, polymerase enzyme and primers; c) the number of cycles of amplification performed; and d) the temperatures and times used at the various steps in the

amplification process [*PCR Technology - Principles and Applications for DNA Amplification* (H.A. Erlich, Ed.), Stockton Press, New York, pp. 7-16 (1989)]. When the specific target sequence is present in low concentration in the sample DNA more non-target fragments are produced. Low target concentration is often the norm in clinical samples where the target may be present as a single copy in the genome or where very little viral DNA is present as in HIV infections.

Because amplification products are produced which do not represent the specific target sequence to be detected, the products of a PCR reaction must be analyzed using a probe specific for the target DNA. The detection of specific amplification products has been accomplished by the hybridization of a probe specific for the target sequence to the reaction products immobilized upon a solid support. Such a detection method is cumbersome and is subject to the same problems associated with the detection of any target molecule by hybridization as discussed above.

A non-hybridization based detection assay for specific PCR products has been described by Holland *et al.*, *Proc. Natl. Acad. Sci. USA* 88:7276 (1991). In this detection system, the 5' nuclease activity of wild type DNA polymerase from *Thermus aquaticus* ("DNAP*Taq*") is used to generate a specific detectable product concomitantly with amplification. An oligonucleotide probe specific for the target DNA is labeled on the 5' end and added to the PCR reaction along with the unlabelled primers used for extension of the target to be amplified. The 5' nuclease activity of the DNAP*Taq* cleaves the labeled probe annealed to the target DNA before the extension of the primer is complete, generating a smaller fragment of the probe. This detection system requires that amplification be performed upon the sample to produce the specific detection product. This is slow and requires cumbersome equipment.

A minimum of 100 starting copies (*i.e.*, copy number prior to amplification) of target DNA were used in this detection system; it is not clear whether fewer starting copies of target DNA will yield detectable results using this method. Very low copy number may be a problem for some clinical samples where very little DNA is obtained due to restrictions on sample size (blood from neonates or fetuses, forensic samples, *etc.*).

While such an assay is an improvement over earlier hybridization detection methods, it still requires that a PCR reaction be performed upon the sample and it possesses certain inherent problems. One such problem is that this system requires that the detection probe must bind to the target DNA before primer extension occurs. If extension occurs first, the probe binding site will be unavailable and no digestion of the probe will occur and therefore no detectable signal will be produced. To overcome this problem the user must vary the relative amounts of primer and probe or manipulate the sequence and length of the probe. The need for such optimization may prove too burdensome for clinical laboratories.

3. Partial Complementarity

Hybridization, regardless of the method used, requires some degree of complementarity between the sequence being assayed (the target sequence) and the fragment of DNA used to perform the test (the probe). (Of course, one can obtain binding without any complementarity but this binding is nonspecific and to be avoided.) For many diagnostic applications, it is not important to determine whether the hybridization represents complete or partial complementarity. For example, where it is desired to detect simply the presence or absence of pathogen DNA (such as from a virus, bacterium, fungi, mycoplasma, protozoan) it is only important that the hybridization method ensures hybridization when the relevant sequence is present; conditions can be selected where both partially complementary probes and completely complementary probes will hybridize. Other diagnostic applications, however, may require that the method of hybridization distinguish between variant target sequences. For example, it may be of interest that a particular allelic variant of a pathogen is present. These normal and variant sequences may differ in one or more bases.

There are other applications that may require that the hybridization method distinguish between partial and complete complementarity. It may be of interest to detect genetic polymorphisms. Human hemoglobin is composed, in part, of four polypeptide chains. Two of these chains are identical chains of 141 amino acids (alpha chains) and two of these chains are identical chains of 146 amino acids (beta chains). The gene encoding the beta chain is known to exhibit polymorphism. The

normal allele encodes a beta chain having glutamic acid at the sixth position. The mutant allele encodes a beta chain having valine at the sixth position. This difference in amino acids has a profound (most profound when the individual is homozygous for the mutant allele) physiological impact known clinically as sickle cell anemia. It is well known that the genetic basis of the amino acid change involves a single base difference between the normal allele DNA sequence and the mutant allele DNA sequence.

Unless combined with other techniques (such as restriction enzyme analysis), hybridization methods that allow for the same level of hybridization in the case of both partial as well as complete complementarity are unsuited for such applications; the probe will hybridize to both the normal and variant target sequence.

Methods have been devised to enable discrimination between partial and complete complementarity. One approach is to take advantage of the temperature requirements of the specific hybridization under study. In typical melting curve experiments, such as those described by Wallace *et al.*, *Nucl. Acids Res.* 6:3543 (1979) and *Nucl. Acids Res.* 9:879 (1981), an immobilized probe-target complex is washed at increasing temperatures under non-equilibrium conditions. It is observed that partially complementary probe-target complexes display a lower thermal stability as compared to completely complementary probe-target complexes. This difference can be used, therefore, to determine whether the probe has hybridized to the partially complementary or the completely complementary target sequence.

Conventional methods that utilize the temperature dependant nature of hybridization are artful. The application of this method for the discrimination of single base mutations in human genomic targets is limited to the use of short oligonucleotide probes where the hybridization interaction with the target sequence is in the size range of 17 bases to 25 bases in length. The lower length limit is determined by the random probability of having a complement to the probe in the human genome, which is greater than 1 for a random 16 base pair interaction, but less than 1 for interactions 17 bases or longer in length. The upper limit is one of practicality. It is difficult to differentiate single base mismatches on the basis of thermal stability for interactions longer than 25 bases in length. These

conventional methods are, unfortunately also time consuming. Probe concentrations in these experiments are approximately 1-5 × 10^{-10}M. These concentrations are empirically derived; they minimize the use of probe and simultaneously provide sufficient discrimination to distinguish single copy genes utilizing probes of approximately 20 nucleotides in length. Hybridization times are two to ten hours at these concentrations. After hybridization, several washes of varying stringency are employed to remove excess probe, non-specifically bound probe, and probe bound to partially complementary sequences in the target genome. Careful control of these wash steps is necessary, since the signal (specifically bound probe) to noise (non-specifically bound probe) ratio of the experiment is ultimately determined by the wash procedures.

No detection method heretofore described has solved all three of the problems discussed above. The PCR process solves the problem of low target concentration. However, the specific detection of PCR products by any hybridization method is subject to the same problems associated with the detection of any target molecules. The detection of single base differences between PCR targets was initially accomplished through the use of a restriction enzyme analysis of the hybridization completes formed between oligonucleotide probes and PCR targets. This technique is limited by that fact that restriction enzymes do not exist for all sequences. More recent studies have achieved discrimination without restriction enzymes, however these studies have involved the inefficient immobilization of target nucleic acids to solid surfaces [dot blot hybridization; Saiki *et al.*, *Nature* 324:163 (1986)].

Another method for the detection of allele-specific variants is disclosed by Kwok *et al.*, *Nucl. Acids Res.* 18:999 (1990). This method is based upon the fact that it is difficult for a DNAP to synthesize a DNA strand when there is a mismatch between the template strand and the primer. The mismatch acts to prevent the extension thereby preventing the amplification of a target DNA that is not perfectly complementary to the primer used in a PCR reaction. While an allele-specific variant may be detected by the use of a primer that is perfectly matched with only one of the possible alleles, this method of detection is artful and has limitations. Particularly troublesome is the fact that the base composition of

the mismatch influences the ability to prevent extension across the mismatch. Certain mismatches do not prevent extension or have only a minimal effect.

An ideal method of detecting specific target DNAs would allow detection without the need to amplify the sample DNA first and would allow the detection of target sequences which are present in low copy numbers in the DNA sample. This ideal method would also allow the discrimination between variants of the target sequence such that single base variations between alleles of mammalian genes can be discerned.

One object of the present invention is to provide a method of detection of specific nucleic acid sequences that solves the above-named problems.

SUMMARY OF THE INVENTION

The present invention relates to means for cleaving a nucleic acid cleavage structure in a site-specific manner. In one embodiment, the means for cleaving is a cleaving enzyme comprising 5' nucleases derived from thermostable DNA polymerases. These polymerases form the basis of a novel method of detection of specific nucleic acid sequences. The present invention contemplates use of the novel detection method for, among other uses, clinical diagnostic purposes.

In one embodiment, the present invention contemplates a DNA sequence encoding a DNA polymerase altered in sequence (*i.e.*, a "mutant" DNA polymerase) relative to the native sequence such that it exhibits altered DNA synthetic activity from that of the native (*i.e.*, "wild type") DNA polymerase. It is preferred that the encoded DNA polymerase is altered such that it exhibits reduced synthetic activity from that of the native DNA polymerase. In this manner, the enzymes of the invention are predominantly 5' nucleases and are capable of cleaving nucleic acids in a structure-specific manner in the absence of interfering synthetic activity.

Importantly, the 5' nucleases of the present invention are capable of cleaving linear duplex structures to create single discrete cleavage products. These linear structures are either 1) not cleaved by the wild type enzymes (to any significant degree), or 2) are cleaved by the wild type enzymes so as to create multiple products. This characteristic of the 5' nucleases has been found to be

consistent of enzymes derived in this manner from thermostable polymerases across eubacterial thermophilic species.

It is not intended that the invention be limited by the nature of the alteration necessary to render the polymerase synthesis deficient nor the extent of the deficiency. The present invention contemplates altered structure (primary, secondary, *etc.*) as well as native structure inhibited by synthesis inhibitors.

Where the structure is altered, it is not intended that the invention be limited by the means by which the structure of the polymerase is altered. In one embodiment, the alteration of the native DNA sequence comprises a change in a single nucleotide. In another embodiment, the alteration of the native DNA sequence comprises a deletion of one or more nucleotides. In yet another embodiment, the alteration of the native DNA sequence comprises an insertion of one or more nucleotides. In either of these cases, the change in DNA sequence may manifest itself in a change in amino acid sequence.

The present invention contemplates 5' nucleases from a variety of sources. The preferred 5' nucleases are thermostable. Thermostable 5' nucleases are contemplated as particularly useful in that they operate at temperatures where nucleic acid hybridization is extremely specific, allowing for allele-specific detection (including single-base mismatches). In one embodiment, the thermostable 5' nucleases are selected from the group consisting of altered polymerases derived from the native polymerases of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

As noted above, the present invention contemplates the use of altered polymerases in a detection method. In one embodiment, the present invention contemplates a method of detecting the presence of a specific target nucleic acid molecule comprising: a) providing: i) a cleavage means, ii) a target nucleic acid, iii) a first oligonucleotide complementary to a first portion of said target nucleic acid, iv) a first solid support having a second oligonucleotide, a region of which is complementary to a second portion of said target nucleic acid, said non-complementary region of said second oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising a first signal oligonucleotide, v) a plurality of "uncleaved" second solid supports each having a third

oligonucleotide, a region of which is complementary to said first signal oligonucleotide, the non-complementary region of said third oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising a second signal oligonucleotide, and vi) a plurality of "uncleaved" third solid supports each having a fourth oligonucleotide, a region of which is complementary to said second signal oligonucleotide, the non-complementary region of said fourth oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising said first signal oligonucleotide; b) mixing said cleavage means, said target nucleic acid, said first oligonucleotide and said second oligonucleotide under conditions wherein said first oligonucleotide and the 3' end of said second oligonucleotide are annealed to said target DNA sequence so as to create a first cleavage structure and cleavage of said first cleavage structure results in the liberating of said first signal oligonucleotide; d) reacting said liberated first signal oligonucleotide with one of said plurality of second solid supports under conditions such that said first signal oligonucleotide hybridizes to said complementary region of said third oligonucleotide to create a second cleavage structure and cleavage of said second cleavage structure results in the liberating of said second signal oligonucleotide and a "cleaved" second solid support; e) reacting said liberated second signal oligonucleotide with one of said plurality of third solid supports under conditions such that said second signal oligonucleotide hybridizes to said complementary region of said fourth oligonucleotide to create a third cleavage structure and cleavage of said third cleavage structure results in the liberating of a second molecule of said first signal oligonucleotide and a "cleaved" third solid support; and h) detecting the presence of said first and second signal oligonucleotides.

It is preferred that, after the hybridization of said first signal oligonucleotide and liberation of said second signal oligonucleotide, said first signal oligonucleotide is itself released from said "cleaved" second solid support and reacted with one of said plurality of "uncleaved" second solid supports. Similarly, it is preferred that, after the hybridization of said second signal oligonucleotide and liberation of said second molecule of said first signal oligonucleotide, said second signal oligonucleotide is itself released from said "cleaved" third solid support and reacted

with on of said plurality of "uncleaved" third solid supports. By the term "cleaved" and "uncleaved" it is not meant to indicate that the solid support (*e.g.*, a bead) is physically cleaved or uncleaved. Rather, it is meant to indicate the status of the oligonucleotide attached to the solid support.

By reference to a "solid support" it is not intended that the invention be limited to separate and discrete supports. For example, the invention contemplates a design where the oligos are on the same solid support, albeit separate in different regions. In one embodiment, the solid support is a microtiter well wherein the oligos are attached (*e.g.*, covalently) or coated (*e.g.*, non-covalently) in different regions of the well.

In a second embodiment, the present invention contemplates a method of detecting the presence of a specific target nucleic acid molecule comprising: a) providing: i) a target nucleic acid, ii) a first oligonucleotide complementary to a first portion of said target nucleic acid, and iii) a second oligonucleotide, a region of which is complementary to a second portion of said target nucleic acid, said non-complementary region of said second oligonucleotide providing a single-stranded arm at its 5' end; b) mixing said target nucleic acid, said first oligonucleotide and said second oligonucleotide under conditions wherein said first oligonucleotide and the 3' end of said second oligonucleotide are annealed to said target DNA sequence so as to create a first cleavage structure; c) providing a cleavage means under conditions such that cleavage of said first cleavage structure occurs preferentially at a site located within said second oligonucleotide in a manner dependent upon the annealing of said first and second oligonucleotides on said target nucleic acid, thereby liberating the single-stranded arm of said second oligonucleotide generating a third oligonucleotide; d) providing a first hairpin structure having a single-stranded 3' arm and a single-stranded 5' arm under conditions wherein said third oligonucleotide anneals to said single-stranded 3' arm of said first hairpin thereby creating a second cleavage structure; e) providing conditions under which cleavage of said second cleavage structure occurs by said cleavage means liberating the single-stranded 5' arm of said second cleavage structure so as to create reaction products comprising a fourth oligonucleotide and a first cleaved hairpin detection molecule; f) providing a second hairpin structure

having a single-stranded 3' arm and a single-stranded 5' arm under conditions wherein said fourth oligonucleotide anneals to the single-stranded 3' arm of said second hairpin thereby creating a third cleavage structure; g) providing conditions under which cleavage of said third cleavage structure occurs by said cleavage means, liberating the single-stranded 5' arm of said third cleavage structure so as to create reaction products comprising generating a fifth oligonucleotide identical in sequence to said third oligonucleotide and a second cleaved hairpin detection molecule; and h) detecting the presence of said first and second cleaved hairpin detection molecules.

In one embodiment, the detection method of the present invention allows the detection of specific target nucleic acid sequences present in a sample without the need to amplify the number of target copies prior to detection. In this embodiment, steps d) through g) of the method are repeated at least once.

In a preferred embodiment, the cleavage means comprises a cleavage enzyme comprising an altered thermostable DNA polymerase having reduced synthesis capability, *i.e.*, a 5' nuclease derived from a thermostable DNA polymerase. While a complete absence of synthesis is not required, it is desired that cleavage reactions occur in the absence of polymerase activity at a level where it interferes with the discrimination needed for detection.

While the cleavage of the second embodiment of the detection method of the present invention can be independent of the annealing of the oligonucleotides, it is preferred that the cleavage is primer-dependent. In other words, it is desired that the cleavage reactions of steps c), e) and g) will not occur absent the annealing of said first oligonucleotide, said third oligonucleotide and said fourth oligonucleotide, respectively.

While cleavage is site-specific, the present invention allows for cleavage at a variety of sites. In one embodiment, the cleavage reaction of step c) occurs within the annealed portion of said second oligonucleotide. In another embodiment, the cleavage reaction of step c) occurs within the non-annealed portion of said second oligonucleotide.

DESCRIPTION OF THE DRAWINGS

Figure 1A provides a schematic of one embodiment of the detection method of the present invention.

Figure 1B provides a schematic of a second embodiment of the detection method of the present invention.

Figure 2 is a comparison of the nucleotide structure of the DNAP genes isolated from *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*; the consensus sequence is shown at the top of each row.

Figure 3 is a comparison of the amino acid sequence of the DNAP isolated from *Thermus aquaticus*, *Thermus flavus*, and *Thermus thermophilus*; the consensus sequence is shown at the top of each row.

Figures 4A-G are a set of diagrams of wild-type and synthesis-deficient DNAP*Taq* genes.

Figure 5A depicts the wild-type *Thermus flavus* polymerase gene.

Figure 5B depicts a synthesis-deficient *Thermus flavus* polymerase gene.

Figure 6 depicts a structure which cannot be amplified using DNAP*Taq*.

Figure 7 is a ethidium bromide-stained gel demonstrating attempts to amplify a bifurcated duplex using either DNAP*Taq* or DNAPStf.

Figure 8 is an autoradiogram of a gel analyzing the cleavage of a bifurcated duplex by DNAP*Taq* and lack of cleavage by DNAPStf.

Figures 9A-B are a set of autoradiograms of gels analyzing cleavage or lack of cleavage upon addition of different reaction components and change of incubation temperature during attempts to cleave a bifurcated duplex with DNAP*Taq*.

Figures 10A-B are an autoradiogram displaying timed cleavage reactions, with and without primer.

Figures 11A-B are a set of autoradiograms of gels demonstrating attempts to cleave a bifurcated duplex (with and without primer) with various DNAPs.

Figures 12A shows the substrates and oligonucleotides used to test the specific cleavage of substrate DNAs targeted by pilot oligonucleotides.

Figure 12B shows an autoradiogram of a gel showing the results of cleavage reactions using the substrates and oligonucleotides shown Fig. 12A.

Figure 13A shows the substrate and oligonucleotide used to test the specific cleavage of a substrate RNA targeted by a pilot oligonucleotide.

Figure 13B shows an autoradiogram of a gel showing the results of a cleavage reaction using the substrate and oligonucleotide shown in Fig. 13A.

Figure 14 is a diagram of vector pTTQ18.

Figure 15 is a diagram of vector pET-3c.

Figure 16A-E depicts a set of molecules which are suitable substrates for cleavage by the 5' nuclease activity of DNAPs.

Figure 17 is an autoradiogram of a gel showing the results of a cleavage reaction run with synthesis-deficient DNAPs.

Figure 18 is an autoradiogram of a PEI chromatogram resolving the products of an assay for synthetic activity in synthesis-deficient DNAP*Taq* clones.

Figure 19A depicts the substrate molecule used to test the ability of synthesis-deficient DNAPs to cleave short hairpin structures.

Figure 19B shows an autoradiogram of a gel resolving the products of a cleavage reaction run using the substrate shown in Fig. 19A.

Figure 20A shows the A- and T-hairpin molecules used in the trigger/detection assay.

Figure 20B shows the sequence of the alpha primer used in the trigger/detection assay.

Figure 20C shows the structure of the cleaved A- and T-hairpin molecules.

Figure 20D depicts the complementarity between the A- and T-hairpin molecules.

Figure 21 provides the complete 206-mer duplex sequence employed as a substrate for the 5' nucleases of the present invention

Figures 22A and B show the cleavage of linear nucleic acid substrates (based on the 206-mer of Figure 21) by wild type DNAPs and 5' nucleases isolated from *Thermus aquaticus* and *Thermus flavus*.

Figure 23 provides a detailed schematic corresponding to the of one embodiment of the detection method of the present invention.

Figure 24 shows the propagation of cleavage of the linear duplex nucleic acid structures of Figure 23 by the 5' nucleases of the present invention.

Figure 25A shows the "nibbling" phenomenon detected with the DNAPs of the present invention.

Figure 25B shows that the "nibbling" of Figure 25A is 5' nucleolytic cleavage and not phosphatase cleavage.

Figure 26 demonstrates that the "nibbling" phenomenon is duplex dependent.

Figure 27 is a schematic showing how "nibbling" can be employed in a detection assay.

Figure 28 demonstrates that "nibbling" can be target directed.

DESCRIPTION OF THE INVENTION

The present invention relates to means for cleaving a nucleic acid cleavage structure in a site-specific manner. In particular, the present invention relates to a cleaving enzyme having 5' nuclease activity without interfering nucleic acid synthetic ability.

This invention provides 5' nucleases derived from thermostable DNA polymerases which exhibit altered DNA synthetic activity from that of native thermostable DNA polymerases. The 5' nuclease activity of the polymerase is retained while the synthetic activity is reduced or absent. Such 5' nucleases are capable of catalyzing the structure-specific cleavage of nucleic acids in the absence of interfering synthetic activity. The lack of synthetic activity during a cleavage reaction results in nucleic acid cleavage products of uniform size.

The novel properties of the polymerases of the invention form the basis of a method of detecting specific nucleic acid sequences. This method relies upon the amplification of the detection molecule rather than upon the amplification of the target sequence itself as do existing methods of detecting specific target sequences.

DNA polymerases (DNAPs), such as those isolated from *E. coli* or from thermophilic bacteria of the genus *Thermus*, are enzymes that synthesize new DNA strands. Several of the known DNAPs contain associated nuclease activities in addition to the synthetic activity of the enzyme.

Some DNAPs are known to remove nucleotides from the 5' and 3' ends of DNA chains [Kornberg, *DNA Replication,* W.H. Freeman and Co., San Francisco,

pp. 127-139 (1980)]. These nuclease activities are usually referred to as 5' exonuclease and 3' exonuclease activities, respectively. For example, the 5' exonuclease activity located in the N-terminal domain of several DNAPs participates in the removal of RNA primers during lagging strand synthesis during DNA replication and the removal of damaged nucleotides during repair. Some DNAPs, such as the *E. coli* DNA polymerase (DNAPEc1), also have a 3' exonuclease activity responsible for proof-reading during DNA synthesis (Kornberg, *supra*).

A DNAP isolated from *Thermus aquaticus*, termed *Taq* DNA polymerase (DNAP*Taq*), has a 5' exonuclease activity, but lacks a functional 3' exonucleolytic domain [Tindall and Kunkell, *Biochem.* 27:6008 (1988)]. Derivatives of DNAPEc1 and DNAP*Taq*, respectively called the Klenow and Stoffel fragments, lack 5' exonuclease domains as a result of enzymatic or genetic manipulations [Brutlag *et al.*, *Biochem. Biophys. Res. Commun.* 37:982 (1969); Erlich *et al.*, *Science* 252:1643 (1991); Setlow and Kornberg, *J. Biol. Chem.* 247:232 (1972)].

The 5' exonuclease activity of DNAP*Taq* was reported to require concurrent synthesis [Gelfand, *PCR Technology - Principles and Applications for DNA Amplification* (H.A. Erlich, Ed.), Stockton Press, New York, p. 19 (1989)]. Although mononucleotides predominate among the digestion products of the 5' exonucleases of DNAP*Taq* and DNAPEc1, short oligonucleotides (≤ 12 nucleotides) can also be observed implying that these so-called 5' exonucleases can function endonucleolytically [Setlow, *supra*; Holland *et al.*, *Proc. Natl. Acad. Sci.* USA 88:7276 (1991)].

In WO 92/06200, Gelfand *et al.* show that the preferred substrate of the 5' exonuclease activity of the thermostable DNA polymerases is displaced single-stranded DNA. Hydrolysis of the phosphodiester bond occurs between the displaced single-stranded DNA and the double-helical DNA with the preferred exonuclease cleavage site being a phosphodiester bond in the double helical region. Thus, the 5' exonuclease activity usually associated with DNAPs is a structure-dependent single-stranded endonuclease and is more properly referred to as a 5' nuclease. Exonucleases are enzymes which cleave nucleotide molecules from the ends of the nucleic acid molecule. Endonucleases, on the other hand, are enzymes

which cleave the nucleic acid molecule at internal rather than terminal sites. The nuclease activity associated with some thermostable DNA polymerases cleaves endonucleolytically but this cleavage requires contact with the 5' end of the molecule being cleaved. Therefore, these nucleases are referred to as 5' nucleases.

When a 5' nuclease activity is associated with a eubacterial Type A DNA polymerase, it is found in the one-third N-terminal region of the protein as an independent functional domain. The C-terminal two-thirds of the molecule constitute the polymerization domain which is responsible for the synthesis of DNA. Some Type A DNA polymerases also have a 3' exonuclease activity associated with the two-third C-terminal region of the molecule.

The 5' exonuclease activity and the polymerization activity of DNAPs have been separated by proteolytic cleavage or genetic manipulation of the polymerase molecule. To date thermostable DNAPs have been modified to remove or reduce the amount of 5' nuclease activity while leaving the polymerase activity intact.

The Klenow or large proteolytic cleavage fragment of DNAPEc1 contains the polymerase and 3' exonuclease activity but lacks the 5' nuclease activity. The Stoffel fragment of DNAP*Taq* lacks the 5' nuclease activity due to a genetic manipulation which deleted the N-terminal 289 amino acids of the polymerase molecule [Erlich *et al.*, *Science* 252:1643 (1991)]. WO 92/06200 describes a thermostable DNAP with an altered level of 5' to 3' exonuclease. U.S. Patent No. 5,108,892 describes a *Thermus aquaticus* DNAP without a 5' to 3' exonuclease. However, the art of molecular biology lacks a thermostable DNA polymerase with a lessened amount of synthetic activity.

The present invention provides 5' nucleases derived from thermostable Type A DNA polymerases that retain 5' nuclease activity but have reduced or absent synthetic activity. The ability to uncouple the synthetic activity of the enzyme from the 5' nuclease activity proves that the 5' nuclease activity does not require concurrent DNA synthesis as was previously reported (Gelfand, *PCR Technology*, *supra*).

The description of the invention is divided into: I. Detection of Specific Nucleic Acid Sequences Using 5' Nucleases; II. Generation of 5' Nucleases Derived From Thermostable DNA Polymerases; III. Therapeutic Uses of 5'

Nucleases; and IV. Detection of Antigenic or Nucleic Acid Targets by a Dual Capture Assay. To facilitate understanding of the invention, a number of terms are defined below.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. In contrast, the term "modified" or mutant" refers to a gene or gene product which displays altered characteristics when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "recombinant DNA vector" as used herein refers to DNA sequences containing a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism. DNA sequences necessary for expression in procaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eucaryotic cells are known to utilize promoters, polyadenlyation signals and enhancers.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phoshodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is

not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when place under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base

composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

Stability of a nucleic acid duplex is measured by the melting temperature, or "T_m." The T_m of a particular nucleic acid duplex under specified conditions is the temperature at which half of the base pairs have disassociated.

The term "probe" as used herein refers to a labeled oligonucleotide which forms a duplex structure with a sequence in another nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the other nucleic acid.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

The term "cleavage structure" as used herein, refers to a nucleic acid structure which is a substrate for cleavage by the 5' nuclease activity of a DNAP.

The term "cleavage means" as used herein refers to any means which is capable of cleaving a cleavage structure in a specific manner. The cleavage means may include native DNAPs having 5' nuclease activity, and, more specifically, modified DNAPs having 5' nuclease but lacking synthetic activity.

The term "liberating" as used herein refers to the release of a nucleic acid fragment from a larger nucleic acid fragment, such as an oligonucleotide, by the action of a 5' nuclease such that the released fragment is no longer covalently attached to the remainder of the oligonucleotide.

The term "substrate strand" as used herein, means that strand of nucleic acid in a cleavage structure in which the cleavage mediated by the 5' nuclease activity occurs.

The term "template strand" as used herein, means that strand of nucleic acid in a cleavage structure which is at least partially complementary to the substrate strand and which anneals to the substrate strand to form the cleavage structure.

The term "K_m" as used herein refers to the Michaelis-Menten constant for an enzyme and is defined as the concentration of the specific substrate at which a

given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction.

I. Detection Of Specific Nucleic Acid Sequences Using 5' Nucleases

The 5' nucleases of the invention form the basis of a novel detection assay for the identification of specific nucleic acid sequences. This detection system identifies the presence of specific nucleic acid sequences by requiring the annealing of two oligonucleotide probes to two portions of the target sequence. As used herein, the term "target sequence" or target nucleic acid sequence" refers to a specific nucleic acid sequence within a polynucleotide sequence, such as genomic DNA or RNA, which is to be either detected or cleaved or both.

Figure 1A provides a schematic of one embodiment of the detection method of the present invention. The target sequence is recognized by two distinct oligonucleotides in the triggering or trigger reaction. It is preferred that one of these oligonucleotides is provided on a solid support. The other can be provided free. In Figure 1A the free oligo is indicated as a "primer" and the other oligo is shown attached to a bead designated as type 1. The target nucleic acid aligns the two oligonucleotides for specific cleavage of the 5' arm (of the oligo on bead 1) by the DNAPs of the present invention (not shown in Figure 1A).

The site of cleavage (indicated by a large solid arrowhead) is controlled by the distance between the 3' end of the "primer" and the downstream fork of the oligo on bead 1. The latter is designed with an uncleavable region (indicated by the striping). In this manner neither oligonucleotide is subject to cleavage when misaligned or when unattached to target nucleic acid.

Successful cleavage releases a single copy of what is referred to as the alpha signal oligo. This oligo may contain a detectable moiety (*e.g.*, fluorescein). On the other hand, it may be unlabelled.

In one embodiment of the detection method, two more oligonucleotides are provided on solid supports. The oligonucleotide shown in Figure 1A on bead 2 has a region that is complementary to the alpha signal oligo (indicated as alpha prime) allowing for hybridization. This structure can be cleaved by the DNAPs of the present invention to release the beta signal oligo. The beta signal oligo can then

hybridize to type 3 beads having an oligo with a complementary region (indicated as beta prime). Again, this structure can be cleaved by the DNAPs of the present invention to release a new alpha oligo.

At this point, the amplification has been linear. To increase the power of the method, it is desired that the alpha signal oligo hybridized to bead type 2 be liberated after release of the beta oligo so that it may go on to hybridize with other oligos on type 2 beads. Similarly, after release of an alpha oligo from type 3 beads, it is desired that the beta oligo be liberated.

The liberation of "captured" signal oligos can be achieved in a number of ways. First, it has been found that the DNAPs of the present invention have a true 5' exonuclease capable of "nibbling" the 5' end of the alpha (and beta) prime oligo (discussed below in more detail). Thus, under appropriate conditions, the hybridization is destabilized by nibbling of the DNAP. Second, the alpha - alpha prime (as well as the beta - beta prime) complex can be destablized by heat (*e.g.*, thermal cycling).

With the liberation of signal oligos by such techniques, each cleavage results in a doubling of the number of signal oligos. In this manner, detectable signal can quickly be achieved.

Figure 1B provides a schematic of a second embodiment of the detection method of the present invention. Again, the target sequence is recognized by two distinct oligonucleotides in the triggering or trigger reaction and the target nucleic acid aligns the two oligonucleotides for specific cleavage of the 5' arm by the DNAPs of the present invention (not shown in Figure 1B). The first oligo is completely complementary to a portion of the target sequence. The second oligonucleotide is partially complementary to the target sequence; the 3' end of the second oligonucleotide is fully complementary to the target sequence while the 5' end is non-complementary and forms a single-stranded arm. The non-complementary end of the second oligonucleotide may be a generic sequence which can be used with a set of standard hairpin structures (described below). The detection of different target sequences would require unique portions of two oligonucleotides: the entire first oligonucleotide and the 3' end of the second

oligonucleotide. The 5' arm of the second oligonucleotide can be invariant or generic in sequence.

The annealing of the first and second oligonucleotides near one another along the target sequence forms a forked cleavage structure which is a substrate for the 5' nuclease of DNA polymerases. The approximate location of the cleavage site is again indicated by the large solid arrowhead in Figure 1B.

The 5' nucleases of the invention are capable of cleaving this structure but are not capable of polymerizing the extension of the 3' end of the first oligonucleotide. The lack of polymerization activity is advantageous as extension of the first oligonucleotide results in displacement of the annealed region of the second oligonucleotide and results in moving the site of cleavage along the second oligonucleotide. If polymerization is allowed to occur to any significant amount, multiple lengths of cleavage product will be generated. A single cleavage product of uniform length is desirable as this cleavage product initiates the detection reaction.

The trigger reaction may be run under conditions that allow for thermocycling. Thermocycling of the reaction allows for a logarithmic increase in the amount of the trigger oligonucleotide released in the reaction.

The second part of the detection method allows the annealing of the fragment of the second oligonucleotide liberated by the cleavage of the first cleavage structure formed in the triggering reaction (called the third or trigger oligonucleotide) to a first hairpin structure. This first hairpin structure has a single-stranded 5' arm and a single-stranded 3' arm. The third oligonucleotide triggers the cleavage of this first hairpin structure by annealing to the 3' arm of the hairpin thereby forming a substrate for cleavage by the 5' nuclease of the present invention. The cleavage of this first hairpin structure generates two reaction products: 1) the cleaved 5' arm of the hairpin called the fourth oligonucleotide, and 2) the cleaved hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved first hairpin may be used as a detection molecule to indicate that cleavage directed by the trigger or third oligonucleotide occurred. Thus, this indicates that the first two oligonucleotides

found and annealed to the target sequence thereby indicating the presence of the target sequence in the sample.

The detection products are amplified by having the fourth oligonucleotide anneal to a second hairpin structure. This hairpin structure has a 5' single-stranded arm and a 3' single-stranded arm. The fourth oligonucleotide generated by cleavage of the first hairpin structure anneals to the 3' arm of the second hairpin structure thereby creating a third cleavage structure recognized by the 5' nuclease. The cleavage of this second hairpin structure also generates two reaction products: 1) the cleaved 5' arm of the hairpin called the fifth oligonucleotide which is similar or identical in sequence to the third nucleotide, and 2) the cleaved second hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved second hairpin may be as a detection molecule and amplifies the signal generated by the cleavage of the first hairpin structure. Simultaneously with the annealing of the forth oligonucleotide, the third oligonucleotide is dissociated from the cleaved first hairpin molecule so that it is free to anneal to a new copy of the first hairpin structure. The disassociation of the oligonucleotides from the hairpin structures may be accomplished by heating or other means suitable to disrupt base-pairing interactions.

Further amplification of the detection signal is achieved by annealing the fifth oligonucleotide (similar or identical in sequence to the third oligonucleotide) to another molecule of the first hairpin structure. Cleavage is then performed and the oligonucleotide that is liberated then is annealed to another molecule of the second hairpin structure. Successive rounds of annealing and cleavage of the first and second hairpin structures, provided in excess, are performed to generate a sufficient amount of cleaved hairpin products to be detected. The temperature of the detection reaction is cycled just below and just above the annealing temperature for the oligonucleotides used to direct cleavage of the hairpin structures, generally about 55°C to 70°C. The number of cleavages will double in each cycle until the amount of hairpin structures remaining is below the K_m for the hairpin structures. This point is reached when the hairpin structures are substantially used up. When the detection reaction is to be used in a quantitative manner, the cycling reactions

are stopped before the accumulation of the cleaved hairpin detection products reach a plateau.

Detection of the cleaved hairpin structures may be achieved in several ways. In one embodiment detection is achieved by separation on agarose or polyacrylamide gels followed by staining with ethidium bromide. In another embodiment, detection is achieved by separation of the cleaved and uncleaved hairpin structures on a gel followed by autoradiography when the hairpin structures are first labelled with a radioactive probe and separation on chromatography columns using HPLC or FPLC followed by detection of the differently sized fragments by absorption at OD_{260} . Other means of detection include detection of changes in fluorescence polarization when the single-stranded 5' arm is released by cleavage, the increase in fluorescence of an intercalating fluorescent indicator as the amount of primers annealed to 3' arms of the hairpin structures increases. The formation of increasing amounts of duplex DNA (between the primer and the 3' arm of the hairpin) occurs if successive rounds of cleavage occur.

The hairpin structures may be attached to a solid support, such as an agarose, styrene or magnetic bead, via the 3' end of the hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead, if so desired. The advantage of attaching the hairpin structures to a solid support is that this prevents the hybridization of the two hairpin structures to one another over regions which are complementary. If the hairpin structures anneal to one another, this would reduce the amount of hairpins available for hybridization to the primers released during the cleavage reactions. If the hairpin structures are attached to a solid support, then additional methods of detection of the products of the cleavage reaction may be employed. These methods include, but are not limited to, the measurement of the released single-stranded 5' arm when the 5' arm contains a label at the 5' terminus. This label may be radioactive, fluorescent, biotinylated, etc. If the hairpin structure is not cleaved, the 5' label will remain attached to the solid support. If cleavage occurs, the 5' label will be released from the solid support.

The 3' end of the hairpin molecule may be blocked through the use of dideoxynucleotides. A 3' terminus containing a dideoxynucleotide is unavailable to

participate in reactions with certain DNA modifying enzymes, such as terminal transferase. Cleavage of the hairpin having a 3' terminal dideoxynucleotide generates a new, unblocked 3' terminus at the site of cleavage. This new 3' end has a free hydroxyl group which can interact with terminal transferase thus providing another means of detecting the cleavage products.

The hairpin structures are designed so that their self-complementary regions are very short (generally in the range of 3-8 base pairs). Thus, the hairpin structures are not stable at the high temperatures at which this reaction is performed (generally in the range of 50-75°C) unless the hairpin is stabilized by the presence of the annealed oligonucleotide on the 3' arm of the hairpin. This instability prevents the polymerase from cleaving the hairpin structure in the absence of an associated primer thereby preventing false positive results due to non-oligonucleotide directed cleavage.

As discussed above, the use of the 5' nucleases of the invention which have reduced polymerization activity is advantageous in this method of detecting specific nucleic acid sequences. Significant amounts of polymerization during the cleavage reaction would cause shifting of the site of cleavage in unpredictable ways resulting in the production of a series of cleaved hairpin structures of various sizes rather than a single easily quantifiable product. Additionally, the primers used in one round of cleavage could, if elongated, become unusable for the next cycle, by either forming an incorrect structure or by being too long to melt off under moderate temperature cycling conditions. In a pristine system (*i.e.*, lacking the presence of dNTPs), one could use the unmodified polymerase, but the presence of nucleotides (dNTPs) can decrease the per cycle efficiency enough to give a false negative result. When a crude extract (genomic DNA preparations, crude cell lysates, *etc.*) is employed or where a sample of DNA from a PCR reaction, or any other sample that might be contaminated with dNTPs, the 5' nucleases of the present invention that were derived from thermostable polymerases are particularly useful.

II. Generation Of 5' Nucleases From Thermostable DNA Polymerases

The genes encoding Type A DNA polymerases share about 85% homology to each other on the DNA sequence level. Preferred examples of thermostable polymerases include those isolated from *Thermus aquaticus*, *Thermus flavus*, and *Thermus thermophilus*. However, other thermostable Type A polymerases which have 5' nuclease activity are also suitable. Figs. 2 and 3 compare the nucleotide and amino acid sequences of the three above mentioned polymerases. SEQ ID NOS:1-3 display the nucleotide sequences and SEQ ID NOS:4-6 display the amino acid sequences of the three wild-type polymerases. SEQ ID NO:1 corresponds to the nucleic acid sequence of the wild type *Thermus aquaticus* DNA polymerase gene isolated from the YT-1 strain [Lawyer *et al.*, *J. Biol. Chem.* 264:6427 (1989)]. SEQ ID NO:2 corresponds to the nucleic acid sequence of the wild type *Thermus flavus* DNA polymerase gene [Akhmetzjanov and Vakhitov, *Nucl. Acids Res.* 20:5839 (1992)]. SEQ ID NO:3 corresponds to the nucleic acid sequence of the wild type *Thermus thermophilus* DNA polymerase gene [Gelfand *et al.*, WO 91/09950 (1991)]. SEQ ID NOS:7-8 depict the consensus nucleotide and amino acid sequences, respectively for the above three DNAPs (also shown on the top row in Figs. 2 and 3).

The 5' nucleases of the invention derived from thermostable polymerases have reduced synthetic ability, but retain substantially the same 5' exonuclease activity as the native DNA polymerase. The term "substantially the same 5' nuclease activity" as used herein means that the 5' nuclease activity of the modified enzyme retains the ability to function as a structure-dependent single-stranded endonuclease but not necessarily at the same rate of cleavage as compared to the unmodified enzyme. Type A DNA polymerases may also be modified so as to produce an enzyme which has increases 5' nuclease activity while having a reduced level of synthetic activity. Modified enzymes having reduced synthetic activity and increased 5' nuclease activity are also envisioned by the present invention.

By the term "reduced synthetic activity" as used herein it is meant that the modified enzyme has less than the level of synthetic activity found in the unmodified or "native" enzyme. The modified enzyme may have no synthetic

activity remaining or may have that level of synthetic activity that will not interfere with the use of the modified enzyme in the detection assay described below. The 5' nucleases of the present invention are advantageous in situations where the cleavage activity of the polymerase is desired, but the synthetic ability is not (such as in the detection assay of the invention).

As noted above, it is not intended that the invention be limited by the nature of the alteration necessary to render the polymerase synthesis deficient. The present invention contemplates a variety of methods, including but not limited to: 1) proteolysis; 2) recombinant constructs (including mutants); and 3) physical and/or chemical modification and/or inhibition.

1. Proteolysis

Thermostable DNA polymerases having a reduced level of synthetic activity are produced by physically cleaving the unmodified enzyme with proteolytic enzymes to produce fragments of the enzyme that are deficient in synthetic activity but retain 5' nuclease activity. Following proteolytic digestion, the resulting fragments are separated by standard chromatographic techniques and assayed for the ability to synthesize DNA and to act as a 5' nuclease. The assays to determine synthetic activity and 5' nuclease activity are described below.

2. Recombinant Constructs

The examples below describe a preferred method for creating a construct encoding a 5' nuclease derived from a thermostable DNA polymerase. As the Type A DNA polymerases are similar in DNA sequence, the cloning strategies employed for the *Thermus aquaticus* and *flavus* polymerases are applicable to other thermostable Type A polymerases. In general, a thermostable DNA polymerase is cloned by isolating genomic DNA using molecular biological methods from a bacteria containing a thermostable Type A DNA polymerase. This genomic DNA is exposed to primers which are capable of amplifying the polymerase gene by PCR.

This amplified polymerase sequence is then subjected to standard deletion processes to delete the polymerase portion of the gene. Suitable deletion processes are described below in the examples.

The example below discusses the strategy used to determine which portions of the DNAP*Taq* polymerase domain could be removed without eliminating the 5' nuclease activity. Deletion of amino acids from the protein can be done either by deletion of the encoding genetic material, or by introduction of a translational stop codon by mutation or frame shift. In addition, proteolytic treatment of the protein molecule can be performed to remove segments of the protein.

In the examples below, specific alterations of the *Taq* gene were: a deletion between nucleotides 1601 and 2502 (the end of the coding region), a 4 nucleotide insertion at position 2043, and deletions between nucleotides 1614 and 1848 and between nucleotides 875 and 1778 (numbering is as in SEQ ID NO:1). These modified sequences are described below in the examples and at SEQ ID NOS:9-12.

Those skilled in the art understand that single base pair changes can be innocuous in terms of enzyme structure and function. Similarly, small additions and deletions can be present without substantially changing the exonuclease or polymerase function of these enzymes.

Other deletions are also suitable to create the 5' nucleases of the present invention. It is preferable that the deletion decrease the polymerase activity of the 5' nucleases to a level at which synthetic activity will not interfere with the use of the 5' nuclease in the detection assay of the invention. Most preferably, the synthetic ability is absent. Modified polymerases are tested for the presence of synthetic and 5' nuclease activity as in assays described below. Thoughtful consideration of these assays allows for the screening of candidate enzymes whose structure is heretofore as yet unknown. In other words, construct "X" can be evaluated according to the protocol described below to determine whether it is a member of the genus of 5' nucleases of the present invention as defined functionally, rather than structurally.

In the example below, the PCR product of the amplified *Thermus aquaticus* genomic DNA did not have the identical nucleotide structure of the native genomic DNA and did not have the same synthetic ability of the original clone. Base pair

changes which result due to the infidelity of DNAP*Taq* during PCR amplification of a polymerase gene are also a method by which the synthetic ability of a polymerase gene may be inactivated. The examples below and Figs. 4A and 5A indicate regions in the native *Thermus aquaticus* and *flavus* DNA polymerases likely to be important for synthetic ability. There are other base pair changes and substitutions that will likely also inactivate the polymerase.

It is not necessary, however, that one start out the process of producing a 5' nuclease from a DNA polymerase with such a mutated amplified product. This is the method by which the examples below were performed to generate the synthesis-deficient DNAP*Taq* mutants, but it is understood by those skilled in the art that a wild-type DNA polymerase sequence may be used as the starting material for the introduction of deletions, insertion and substitutions to produce a 5' nuclease. For example, to generate the synthesis-deficient DNAPTfl mutant, the primers listed in SEQ ID NOS:13-14 were used to amplify the wild type DNA polymerase gene from *Thermus flavus* strain AT-62. The amplified polymerase gene was then subjected to restriction enzyme digestion to delete a large portion of the domain encoding the synthetic activity.

The present invention contemplates that the nucleic acid construct of the present invention be capable of expression in a suitable host. Those in the art know methods for attaching various promoters and 3' sequences to a gene structure to achieve efficient expression. The examples below disclose two suitable vectors and six suitable vector constructs. Of course, there are other promoter/vector combinations that would be suitable. It is not necessary that a host organism be used for the expression of the nucleic acid constructs of the invention. For example, expression of the protein encoded by a nucleic acid construct may be achieved through the use of a cell-free in vitro transcription/translation system. An example of such a cell-free system is the commercially available TnT™ Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI).

Once a suitable nucleic acid construct has been made, the 5' nuclease may be produced from the construct. The examples below and standard molecular biological teachings enable one to manipulate the construct by different suitable methods.

Once the 5' nuclease has been expressed, the polymerase is tested for both synthetic and nuclease activity as described below.

3. Physical And/Or Chemical Modification And/Or Inhibition

The synthetic activity of a thermostable DNA polymerase may be reduced by chemical and/or physical means. In one embodiment, the cleavage reaction catalyzed by the 5' nuclease activity of the polymerase is run under conditions which preferentially inhibit the synthetic activity of the polymerase. The level of synthetic activity need only be reduced to that level of activity which does not interfere with cleavage reactions requiring no significant synthetic activity.

As shown in the examples below, concentrations of Mg^{++} greater than 5 mM inhibit the polymerization activity of the native DNAP*Taq*. The ability of the 5' nuclease to function under conditions where synthetic activity is inhibited is tested by running the assays for synthetic and 5' nuclease activity, described below, in the presence of a range of Mg^{++} concentrations (5 to 10 mM). The effect of a given concentration of Mg^{++} is determined by quantitation of the amount of synthesis and cleavage in the test reaction as compared to the standard reaction for each assay.

The inhibitory effect of other ions, polyamines, denaturants, such as urea, formamide, dimethylsulfoxide, glycerol and non-ionic detergents (Triton X-100 and Tween-20), nucleic acid binding chemicals such as, actinomycin D, ethidium bromide and psoralens, are tested by their addition to the standard reaction buffers for the synthesis and 5' nuclease assays. Those compounds having a preferential inhibitory effect on the synthetic activity of a thermostable polymerase are then used to create reaction conditions under which 5' nuclease activity (cleavage) is retained while synthetic activity is reduced or eliminated.

Physical means may be used to preferentially inhibit the synthetic activity of a polymerase. For example, the synthetic activity of thermostable polymerases is destroyed by exposure of the polymerase to extreme heat (typically 96 to 100°C) for extended periods of time (greater than or equal to 20 minutes). While these are minor differences with respect to the specific heat tolerance for each of the enzymes, these are readily determined. Polymerases are treated with heat for

various periods of time and the effect of the heat treatment upon the synthetic and 5' nuclease activities is determined.

III. Therapeutic Utility Of 5' Nucleases

The 5' nucleases of the invention have not only the diagnostic utility discussed above, but additionally have therapeutic utility for the cleavage and inactivation of specific mRNAs inside infected cells. The mRNAs of pathogenic agents, such as viruses, bacteria, are targeted for cleavage by a synthesis-deficient DNA polymerase by the introduction of a oligonucleotide complementary to a given mRNA produced by the pathogenic agent into the infected cell along with the synthesis-deficient polymerase. Any pathogenic agent may be targeted by this method provided the nucleotide sequence information is available so that an appropriate oligonucleotide may be synthesized. The synthetic oligonucleotide anneals to the complementary mRNA thereby forming a cleavage structure recognized by the modified enzyme. The ability of the 5' nuclease activity of thermostable DNA polymerases to cleave RNA-DNA hybrids is shown herein in Example 1D.

Liposomes provide a convenient delivery system. The synthetic oligonucleotide may be conjugated or bound to the nuclease to allow for co-delivery of these molecules. Additional delivery systems may be employed.

Inactivation of pathogenic mRNAs has been described using antisense gene regulation and using ribozymes (Rossi, U.S. Patent No. 5,144,019, hereby incorporated by reference). Both of these methodologies have limitations.

The use of antisense RNA to impair gene expression requires stoichiometric and therefore, large molar excesses of anti-sense RNA relative to the pathogenic RNA to be effective. Ribozyme therapy, on the other hand, is catalytic and therefore lacks the problem of the need for a large molar excess of the therapeutic compound found with antisense methods. However, ribozyme cleavage of a given RNA requires the presence of highly conserved sequences to form the catalytically active cleavage structure. This requires that the target pathogenic mRNA contain the conserved sequences (GAAAC $(X)_n$ GU) thereby limiting the number of pathogenic mRNAs that can be cleaved by this method. In contrast, the catalytic

cleavage of RNA by the use of a DNA oligonucleotide and a 5' nuclease is dependent upon structure only; thus, virtually any pathogenic RNA sequence can be used to design an appropriate cleavage structure.

IV. Detection Of Antigenic Or Nucleic Acid Targets By A Dual Capture Assay

The ability to generate 5' nucleases from thermostable DNA polymerases provides the basis for a novel means of detecting the presence of antigenic or nucleic acid targets. In this dual capture assay, the polymerase domains encoding the synthetic activity and the nuclease activity are covalently attached to two separate and distinct antibodies or oligonucleotides. When both the synthetic and the nuclease domains are present in the same reaction and dATP, dTTP and a small amount of poly d(A-T) are provided, an enormous amount of poly d(A-T) is produced. The large amounts of poly d(A-T) are produced as a result of the ability of the 5' nuclease to cleave newly made poly d(A-T) to generate primers that are, in turn, used by the synthetic domain to catalyze the production of even more poly d(A-T). The 5' nuclease is able to cleave poly d(A-T) because poly d(A-T) is self-complementary and easily forms alternate structures at elevated temperatures. These structures are recognized by the 5' nuclease and are then cleaved to generate more primer for the synthesis reaction.

The following is an example of the dual capture assay to detect an antigen(s): A sample to be analyzed for a given antigen(s) is provided. This sample may comprise a mixture of cells; for example, cells infected with viruses display virally-encoded antigens on their surface. If the antigen(s) to be detected are present in solution, they are first attached to a solid support such as the wall of a microtiter dish or to a bead using conventional methodologies. The sample is then mixed with 1) the synthetic domain of a thermostable DNA polymerase conjugated to an antibody which recognizes either a first antigen or a first epitope on an antigen, and 2) the 5' nuclease domain of a thermostable DNA polymerase conjugated to a second antibody which recognizes either a second, distinct antigen or a second epitope on the same antigen as recognized by the antibody conjugated to the synthetic domain. Following an appropriate period to allow the interaction

of the antibodies with their cognate antigens (conditions will vary depending upon the antibodies used; appropriate conditions are well known in the art), the sample is then washed to remove unbound antibody-enzyme domain complexes. dATP, dTTP and a small amount of poly d(A-T) is then added to the washed sample and the sample is incubated at elevated temperatures (generally in the range of 60-80°C and more preferably, 70-75°C) to permit the thermostable synthetic and 5' nuclease domains to function. If the sample contains the antigen(s) recognized by both separately conjugated domains of the polymerase, then an exponential increase in poly d(A-T) production occurs. If only the antibody conjugated to the synthetic domain of the polymerase is present in the sample such that no 5' nuclease domain is present in the washed sample, then only an arithmetic increase in poly d(A-T) is possible. The reaction conditions may be controlled in such a way so that an arithmetic increase in poly d(A-T) is below the threshold of detection. This may be accomplished by controlling the length of time the reaction is allowed to proceed or by adding so little poly d(A-T) to act as template that in the absence of nuclease activity to generate new poly d(A-T) primers very little poly d(A-T) is synthesized.

It is not necessary for both domains of the enzyme to be conjugated to an antibody. One can provide the synthetic domain conjugated to an antibody and provide the 5' nuclease domain in solution or vice versa. In such a case the conjugated antibody-enzyme domain is added to the sample, incubated, then washed. dATP, dTTP, poly d(A-T) and the remaining enzyme domain in solution is then added.

Additionally, the two enzyme domains may be conjugated to oligonucleotides such that target nucleic acid sequences can be detected. The oligonucleotides conjugated to the two different enzyme domains may recognize different regions on the same target nucleic acid strand or may recognize two unrelated target nucleic acids.

The production of poly d(A-T) may be detected in many ways including: 1) use of a radioactive label on either the dATP or dTTP supplied for the synthesis of the poly d(A-T), followed by size separation of the reaction products and autoradiography; 2) use of a fluorescent probe on the dATP and a biotinylated

probe on the dTTP supplied for the synthesis of the poly d(A-T), followed by passage of the reaction products over an avidin bead, such as magnetic beads conjugated to avidin; the presence of the florescent probe on the avidin-containing bead indicates that poly d(A-T) has been formed as the fluorescent probe will stick to the avidin bead only if the fluorescenated dATP is incorporated into a covalent linkage with the biotinylated dTTP; and 3) changes fluorescence polarization indicating an increase in size. Other means of detecting the presence of poly d(A-T) include the use of intercalating fluorescence indicators to monitor the increase in duplex DNA formation.

The advantages of the above dual capture assay for detecting antigenic or nucleic acid targets include:

1) No thermocycling of the sample is required. The polymerase domains and the dATP and dTTP are incubated at a fixed temperature (generally about 70°C). After 30 minutes of incubation up to 75% of the added dNTPs are incorporated into poly d(A-T). The lack of thermocycling makes this assay well suited to clinical laboratory settings; there is no need to purchase a thermocycling apparatus and there is no need to maintain very precise temperature control.

2) The reaction conditions are simple. The incubation of the bound enzymatic domains is done in a buffer containing 0.5 mM $MgCl_2$ (higher concentrations may be used), 2-10 mM Tris-Cl, pH 8.5, approximately 50 μM dATP and dTTP. The reaction volume is 10-20 μl and reaction products are detectable within 10-20 minutes.

3) No reaction is detected unless both the synthetic and nuclease activities are present. Thus, a positive result indicates that both probes (antibody or oligonucleotide) have recognized their targets thereby increasing the specificity of recognition by having two different probes bind to the target.

The ability to separate the two enzymatic activities of the DNAP allows for exponential increases in poly d(A-T) production. If a DNAP is used which lacks 5' nuclease activity, such as te Klenow fragment of DNAPEc1, only a linear or arithmetic increase in poly d(A-T) production is possible [Setlow *et al.*, J. Biol. Chem. 247:224 (1972)]. The ability to provide an enzyme having 5' nuclease

activity but lacking synthetic activity is made possible by the disclosure of this invention.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply:°C (degrees Centigrade); g (gravitational field); vol (volume); w/v (weight to volume); v/v (volume to volume); BSA (bovine serum albumin); CTAB (cetyltrimethylammonium bromide); HPLC (high pressure liquid chromatography); DNA (deoxyribonucleic acid); p (plasmid); µl (microliters); ml (milliliters); µg (micrograms); pmoles (picomoles); mg (milligrams); M (molar); mM (milliMolar); µM (microMolar); nm (nanometers); kdal (kilodaltons); OD (optical density); EDTA (ethylene diamine tetra-acetic acid); FITC (fluorescein isothiocyanate); SDS (sodium dodecyl sulfate); $NaPO_4$ (sodium phosphate); Tris (tris(hydroxymethyl)-aminomethane); PMSF (phenylmethylsulfonylfluoride); TBE (Tris-Borate-EDTA, *i.e.*, Tris buffer titrated with boric acid rather than HCl and containing EDTA) ; PBS (phosphate buffered saline); PPBS (phosphate buffered saline containing 1 mM PMSF); PAGE (polyacrylamide gel electrophoresis); Tween (polyoxyethylene-sorbitan); Dynal (Dynal A.S., Oslo, Norway); Epicentre (Epicentre Technologies, Madison, WI); National Biosciences (Plymouth, MN); New England Biolabs (Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Perkin Elmer (Norwalk, CT); Promega Corp. (Madison, WI); Stratagene (Stratagene Cloning Systems, La Jolla, CA); USB (U.S. Biochemical, Cleveland, OH).

EXAMPLE 1

Characteristics Of Native Thermostable DNA Polymerases

A. 5' Nuclease Activity Of DNAP*Taq*

During the polymerase chain reaction (PCR) [Saiki *et al.*, *Science* 239:487 (1988); Mullis and Faloona, *Methods in Enzymology* 155:335 (1987)], DNAP*Taq* is able to amplify many, but not all, DNA sequences. One sequence that cannot be amplified using DNAP*Taq* is shown in Figure 6 (Hairpin structure is SEQ ID NO:15, PRIMERS are SEQ ID NOS:16-17.) This DNA sequence has the distinguishing characteristic of being able to fold on itself to form a hairpin with two single-stranded arms, which correspond to the primers used in PCR.

To test whether this failure to amplify is due to the 5' nuclease activity of the enzyme, we compared the abilities of DNAP*Taq* and DNAPStf to amplify this DNA sequence during 30 cycles of PCR. Synthetic oligonucleotides were obtained from The Biotechnology Center at the University of Wisconsin-Madison. The DNAP*Taq* and DNAPStf were from Perkin Elmer (*i.e.*, Amplitaq™ DNA polymerase and the Stoffel fragment of Amplitaq™ DNA polymerase). The substrate DNA comprised the hairpin structure shown in Figure 6 cloned in a double-stranded form into pUC19. The primers used in the amplification are listed as SEQ ID NOS:16-17. Primer SEQ ID NO:17 is shown annealed to the 3' arm of the hairpin structure in Fig. 6. Primer SEQ ID NO:16 is shown as the first 20 nucleotides in bold on the 5' arm of the hairpin in Fig. 6.

Polymerase chain reactions comprised 1 ng of supercoiled plasmid target DNA, 5 pmoles of each primer, 40 μM each dNTP, and 2.5 units of DNAP*Taq* or DNAPStf, in a 50 μl solution of 10 mM Tris•Cl pH 8.3. The DNAP*Taq* reactions included 50 mM KCl and 1.5 mM $MgCl_2$. The temperature profile was 95°C for 30 sec., 55°C for 1 min. and 72°C for 1 min., through 30 cycles. Ten percent of each reaction was analyzed by gel electrophoresis through 6% polyacrylamide (cross-linked 29:1) in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA.

The results are shown in Figure 7. The expected product was made by DNAPStf (indicated simply as "S") but not by DNAP*Taq* (indicated as "T"). We

conclude that the 5' nuclease activity of DNAP*Taq* is responsible for the lack of amplification of this DNA sequence.

To test whether the 5' unpaired nucleotides in the substrate region of this structured DNA are removed by DNAP*Taq*, the fate of the end-labeled 5' arm during four cycles of PCR was compared using the same two polymerases (Figure. 8). The hairpin templates, such as the one described in Figure 6, were made using DNAPStf and a ^{32}P-5'-end-labeled primer. The 5'-end of the DNA was released as a few large fragments by DNAP*Taq* but not by DNAPStf. The sizes of these fragments (based on their mobilities) show that they contain most or all of the unpaired 5' arm of the DNA. Thus, cleavage occurs at or near the base of the bifurcated duplex. These released fragments terminate with 3' OH groups, as evidenced by direct sequence analysis, and the abilities of the fragments to be extended by terminal deoxynucleotidyl transferase.

Figures 9-11 show the results of experiments designed to characterize the cleavage reaction catalyzed by DNAP*Taq*. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer (complementary to the 3' arm) and 0.5 units of DNAP*Taq* (estimated to be 0.026 pmoles) in a total volume of 10µl of 10 mM Tris-Cl, ph 8.5, 50 mM KCl and 1.5 mM $MgCl_2$. As indicated, some reactions had different concentrations of KCl, and the precise times and temperatures used in each experiment are indicated in the individual figures. The reactions that included a primer used the one shown in Figure 6 (SEQ ID NO:17). In some instances, the primer was extended to the junction site by providing polymerase and selected nucleotides.

Reactions were initiated at the final reaction temperature by the addition of either the $MgCl_2$ or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. The T_m calculations listed were made using the Oligo™ primer analysis software from National Biosciences, Inc. These were determined using 0.25 µM as the DNA concentration, at either 15 or 65 mM total salt (the 1.5 mM $MgCl_2$ in all reactions was given the value of 15 mM salt for these calculations).

Figure 9 is an autoradiogram containing the results of a set of experiments and conditions on the cleavage site. Figure 9A is a determination of reaction components that enable cleavage. Incubation of 5'-end-labeled hairpin DNA was for 30 minutes at 55°C, with the indicated components. The products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. Figure 9B describes the effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence of KCl for 10 minutes at the indicated temperatures. The lengths of the products, in nucleotides, are indicated.

Surprisingly, cleavage by DNAP*Taq* requires neither a primer nor dNTPs (*see* Fig. 9A). Thus, the 5' nuclease activity can be uncoupled from polymerization. Nuclease activity requires magnesium ions, though manganese ions can be substituted, albeit with potential changes in specificity and activity. Neither zinc nor calcium ions support the cleavage reaction. The reaction occurs over a broad temperature range, from 25°C to 85°C, with the rate of cleavage increasing at higher temperatures.

Still referring to Figure 9, the primer is not elongated in the absence of added dNTPs. However, the primer influences both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (Fig. 9A) apparently results from disruption of a short duplex formed between the arms of the DNA substrate. In the absence of primer, the sequences indicated by underlining in Figure 6 could pair, forming an extended duplex. Cleavage at the end of the extended duplex would release the 11 nucleotide fragment seen on the Fig. 9A lanes with no added primer. Addition of excess primer (Fig. 9A, lanes 3 and 4) or incubation at an elevated temperature (Fig. 9B) disrupts the short extension of the duplex and results in a longer 5' arm and, hence, longer cleavage products.

The location of the 3' end of the primer can influence the precise site of cleavage. Electrophoretic analysis revealed that in the absence of primer (Fig. 9B), cleavage occurs at the end of the substrate duplex (either the extended or shortened form, depending on the temperature) between the first and second base pairs. When the primer extends up to the base of the duplex, cleavage also occurs one nucleotide into the duplex. However, when a gap of four or six nucleotides exists

between the 3' end of the primer and the substrate duplex, the cleavage site is shifted four to six nucleotides in the 5' direction.

Fig. 10 describes the kinetics of cleavage in the presence (Fig. 10A) or absence (Fig. 10B) of a primer oligonucleotide. The reactions were run at 55°C with either 50 mM KCl (Fig. 10A) or 20 mM KCl (Fig. 10B). The reaction products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. "M", indicating a marker, is a 5' end-labeled 19-nt oligonucleotide. Under these salt conditions, Figs. 10A and 10B indicate that the reaction appears to be about twenty times faster in the presence of primer than in the absence of primer. This effect on the efficiency may be attributable to proper alignment and stabilization of the enzyme on the substrate.

The relative influence of primer on cleavage rates becomes much greater when both reactions are run in 50 mM KCl. In the presence of primer, the rate of cleavage increases with KCl concentration, up to about 50 mM. However, inhibition of this reaction in the presence of primer is apparent at 100 mM and is complete at 150 mM KCl. In contrast, in the absence of primer the rate is enhanced by concentration of KCl up to 20 mM, but it is reduced at concentrations above 30 mM. At 50 mM KCl, the reaction is almost completely inhibited. The inhibition of cleavage by KCl in the absence of primer is affected by temperature, being more pronounced at lower temperatures.

Recognition of the 5' end of the arm to be cut appears to be an important feature of substrate recognition. Substrates that lack a free 5' end, such as circular M13 DNA, cannot be cleaved under any conditions tested. Even with substrates having defined 5' arms, the rate of cleavage by DNAP*Taq* is influenced by the length of the arm. In the presence of primer and 50 mM KCl, cleavage of a 5' extension that is 27 nucleotides long is essentially complete within 2 minutes at 55°C. In contrast, cleavages of molecules with 5' arms of 84 and 188 nucleotides are only about 90% and 40% complete after 20 minutes. Incubation at higher temperatures reduces the inhibitory effects of long extensions indicating that secondary structure in the 5' arm or a heat-labile structure in the enzyme may inhibit the reaction. A mixing experiment, run under conditions of substrate

excess, shows that the molecules with long arms do not preferentially tie up the available enzyme in non-productive complexes. These results may indicate that the 5' nuclease domain gains access to the cleavage site at the end of the bifurcated duplex by moving down the 5' arm from one end to the other. Longer 5' arms would be expected to have more adventitious secondary structures (particularly when KCl concentrations are high), which would be likely to impede this movement.

Cleavage does not appear to be inhibited by long 3' arms of either the substrate strand target molecule or pilot nucleic acid, at least up to 2 kilobases. At the other extreme, 3' arms of the pilot nucleic acid as short as one nucleotide can support cleavage in a primer-independent reaction, albeit inefficiently. Fully paired oligonucleotides do not elicit cleavage of DNA templates during primer extension.

The ability of DNAP*Taq* to cleave molecules even when the complementary strand contains only one unpaired 3' nucleotide may be useful in optimizing allele-specific PCR. PCR primers that have unpaired 3' ends could act as pilot oligonucleotides to direct selective cleavage of unwanted templates during preincubation of potential template-primer complexes with DNAP*Taq* in the absence of nucleoside triphosphates.

B. 5' Nuclease Activities Of Other DNAPs

To determine whether other 5' nucleases in other DNAPs would be suitable for the present invention, an array of enzymes, several of which were reported in the literature to be free of apparent 5' nuclease activity, were examined. The ability of these other enzymes to cleave nucleic acids in a structure-specific manner was tested using the hairpin substrate shown in Fig. 6 under conditions reported to be optimal for synthesis by each enzyme.

DNAPEcl and DNAP Klenow were obtained from Promega Corporation; the DNAP of *Pyrococcus furious* ["Pfu", Bargseid *et al.*, Strategies 4:34 (1991)] was from Strategene; the DNAP of *Thermococcus litoralis* ["Tli", Vent™(exo-), Perler *et al.*, Proc. Natl. Acad. Sci. USA 89:5577 (1992)] was from New England Biolabs; the DNAP of *Thermus flavus* ["Tfl", Kaledin *et al.*, *Biokhimiya* 46:1576 (1981)] was from Epicentre Technologies; and the DNAP of *Thermus thermophilus*

["Tth", Carballeira *et al.*, Biotechniques 9:276 (1990); Myers *et al.*, *Biochem.* 30:7661 (1991)] was from U.S. Biochemicals.

0.5 units of each DNA polymerase was assayed in a 20 μl reaction, using either the buffers supplied by the manufacturers for the primer-dependent reactions, or 10 mM Tris•Cl, pH 8.5, 1.5 mM $MgCl_2$, and 20mM KCl. Reaction mixtures were at held 72°C before the addition of enzyme.

Fig. 11 is an autoradiogram recording the results of these tests. Fig. 11A demonstrates reactions of endonucleases of DNAPs of several thermophilic bacteria. The reactions were incubated at 55°C for 10 minutes in the presence of primer or at 72°C for 30 minutes in the absence of primer, and the products were resolved by denaturing polyacrylamide gel electrophoresis. The lengths of the products, in nucleotides, are indicated. Fig. 11B demonstrates endonucleolytic cleavage by the 5' nuclease of DNAPEcl. The DNAPEcl and DNAP Klenow reactions were incubated for 5 minutes at 37°C. Note the light band of cleavage products of 25 and 11 nucleotides in the DNAPEcl lanes (made in the presence and absence of primer, respectively). Fig. 7B also demonstrates DNAP*Taq* reactions in the presence (+) or absence (-) of primer. These reactions were run in 50 mM and 20 mM KCl, respectively, and were incubated at 55°C for 10 minutes.

Referring to Fig. 11A, DNAPs from the eubacteria *Thermus thermophilus* and *Thermus flavus* cleave the substrate at the same place as DNAP*Taq*, both in the presence and absence of primer. In contrast, DNAPs from the archaebacteria *Pyrococcus furiosus* and *Thermococcus litoralis* are unable to cleave the substrates endonucleolytically. The DNAPs from *Pyrococcus furious* and *Thermococcus litoralis* share little sequence homology with eubacterial enzymes (Ito *et al.*, *Nucl. Acids Res.* 19:4045 (1991); Mathur *et al.*, *Nucl. Acids. Res.* 19:6952 (1991); *see also* Perler *et al.*). Referring to Fig. 11B, DNAPEcl also cleaves the substrate, but the resulting cleavage products are difficult to detect unless the 3' exonuclease is inhibited. The amino acid sequences of the 5' nuclease domains of DNAPEcl and DNAP*Taq* are about 38% homologous (Gelfand, *supra*).

The 5' nuclease domain of DNAP*Taq* also shares about 19% homology with the 5' exonuclease encoded by gene 6 of bacteriophage T7 [Dunn *et al.*, *J. Mol. Biol.* 166:477 (1983)]. This nuclease, which is not covalently attached to a DNAP

polymerization domain, is also able to cleave DNA endonucleolytically, at a site similar or identical to the site that is cut by the 5' nucleases described above, in the absence of added primers.

C. Transcleavage

The ability of a 5' nuclease to be directed to cleave efficiently at any specific sequence was demonstrated in the following experiment. A partially complementary oligonucleotide termed a "pilot oligonucleotide" was hybridized to sequences at the desired point of cleavage. The non-complementary part of the pilot oligonucleotide provided a structure analogous to the 3' arm of the template (*see* Fig. 6), whereas the 5' region of the substrate strand became the 5' arm. A primer was provided by designing the 3' region of the pilot so that it would fold on itself creating a short hairpin with a stabilizing tetra-loop [Antao *et al.*, *Nucl. Acids Res.* 19:5901 (1991)]. Two pilot oligonucleotides are shown in Fig. 12A. Oligonucleotides 19-12 (SEQ ID NO:18), 30-12 (SEQ ID NO:19) and 30-0 (SEQ ID NO:40) are 31, 42 or 30 nucleotides long, respectively. However, oligonucleotides 19-12 (SEQ ID NO:18) and 34-19 (SEQ ID NO:19) have only 19 and 30 nucleotides, respectively, that are complementary to different sequences in the substrate strand. The pilot oligonucleotides are calculated to melt off their complements at about 50°C (19-12) and about 75°C (30-12). Both pilots have 12 nucleotides at their 3' ends, which act as 3' arms with base-paired primers attached.

To demonstrate that cleavage could be directed by a pilot oligonucleotide, we incubated a single-stranded target DNA with DNAP*Taq* in the presence of two potential pilot oligonucleotides. The transcleavage reactions, where the target and pilot nucleic acids are not covalently linked, includes 0.01 pmoles of single end-labeled substrate DNA, 1 unit of DNAP*Taq* and 5 pmoles of pilot oligonucleotide in a volume of 20 µl of the same buffers. These components were combined during a one minute incubation at 95°C, to denature the PCR-generated double-stranded substrate DNA, and the temperatures of the reactions were then reduced to their final incubation temperatures. Oligonucleotides 30-12 and 19-12 can hybridize to regions of the substrate DNAs that are 85 and 27 nucleotides from the 5' end of the targeted strand.

Figure 21 shows the complete 206-mer sequence (SEQ ID NO:32). The 206-mer was generated by PCR . The M13/pUC 24-mer reverse sequencing (-48) primer and the M13/pUC sequencing (-47) primer from New England Biolabs (catalogue nos. 1233 and 1224 respectively) were used (50 pmoles each) with the pGEM3z(f+) plasmid vector (Promega Corp.) as template (10 ng) containing the target sequences. The conditions for PCR were as follows: 50 μM of each dNTP and 2.5 units of Taq DNA polymerase in 100 μl of 20 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl with 0.05% Tween-20 and 0.05% NP-40. Reactions were cycled 35 times through 95°C for 45 seconds, 63°C for 45 seconds, then 72°C for 75 seconds. After cycling, reactions were finished off with an incubation at 72°C for 5 minutes. The resulting fragment was purified by electrophoresis through a 6% polyacrylamide gel (29:1 cross link) in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA, visualized by ethidium bromide staining or autoradiography, excised from the gel, eluted by passive diffusion, and concentrated by ethanol precipitation.

Cleavage of the substrate DNA occurred in the presence of the pilot oligonucleotide 19-12 at 50°C (Fig. 12B, lanes 1 and 7) but not at 75°C (lanes 4 and 10). In the presence of oligonucleotide 30-12 cleavage was observed at both temperatures. Cleavage did not occur in the absence of added oligonucleotides (lanes 3, 6 and 12) or at about 80°C even though at 50°C adventitious structures in the substrate allowed primer-independent cleavage in the absence of KCl (Fig. 12B, lane 9). A non-specific oligonucleotide with no complementarity to the substrate DNA did not direct cleavage at 50°C, either in the absence or presence of 50 mM KCl (lanes 13 and 14). Thus, the specificity of the cleavage reactions can be controlled by the extent of complementarity to the substrate and by the conditions of incubation.

D. Cleavage Of RNA

An shortened RNA version of the sequence used in the transcleavage experiments discussed above was tested for its ability to serve as a substrate in the reaction. The RNA is cleaved at the expected place, in a reaction that is dependent upon the presence of the pilot oligonucleotide. The RNA substrate, made by T7 RNA polymerase in the presence of [α-^{32}P]UTP, corresponds to a truncated version of the DNA substrate used in Figure 12B. Reaction conditions were similar to those in used for the DNA substrates described above, with 50 mM KCl; incubation was for 40 minutes at 55°C. The pilot oligonucleotide used is termed 30-0 (SEQ ID NO:20) and is shown in Fig. 13A.

The results of the cleavage reaction is shown in Figure 13B. The reaction was run either in the presence or absence of DNAP*Taq* or pilot oligonucleotide as indicated in Figure 13B.

Strikingly, in the case of RNA cleavage, a 3' arm is not required for the pilot oligonucleotide. It is very unlikely that this cleavage is due to previously described RNaseH, which would be expected to cut the RNA in several places along the 30 base-pair long RNA-DNA duplex. The 5' nuclease of DNAP*Taq* is a structure-specific RNaseH that cleaves the RNA at a single site near the 5' end of the heteroduplexed region.

It is surprising that an oligonucleotide lacking a 3' arm is able to act as a pilot in directing efficient cleavage of an RNA target because such oligonucleotides are unable to direct efficient cleavage of DNA targets using native DNAPs. However, some 5' nucleases of the present invention (for example, clones E, F and G of Figure 4) can cleave DNA in the absence of a 3' arm. In other words, a non-extendable cleavage structure is not required for specific cleavage with some 5' nucleases of the present invention derived from thermostable DNA polymerases.

We tested whether cleavage of an RNA template by DNAP*Taq* in the presence of a fully complementary primer could help explain why DNAP*Taq* is unable to extend a DNA oligonucleotide on an RNA template, in a reaction resembling that of reverse transcriptase. Another thermophilic DNAP, DNAPTth, is able to use RNA as a template, but only in the presence of Mn++, so we predicted that this enzyme would not cleave RNA in the presence of this cation.

Accordingly, we incubated an RNA molecule with an appropriate pilot oligonucleotide in the presence of DNAP*Taq* or DNAPTth, in buffer containing either Mg++ or Mn++. As expected, both enzymes cleaved the RNA in the presence of Mg++. However, DNAP*Taq*, but not DNAPTth, degraded the RNA in the presence of Mn++. We conclude that the 5' nuclease activities of many DNAPs may contribute to their inability to use RNA as templates.

EXAMPLE 2

Generation Of 5' Nucleases From Thermostable DNA Polymerases

Thermostable DNA polymerases were generated which have reduced synthetic activity, an activity that is an undesirable side-reaction during DNA cleavage in the detection assay of the invention, yet have maintained thermostable nuclease activity. The result is a thermostable polymerase which cleaves nucleic acids DNA with extreme specificity.

Type A DNA polymerases from eubacteria of the genus *Thermus* share extensive protein sequence identity (90% in the polymerization domain, using the Lipman-Pearson method in the DNA analysis software from DNAStar, WI) and behave similarly in both polymerization and nuclease assays. Therefore, we have used the genes for the DNA polymerase of *Thermus aquaticus* (DNAP*Taq*) and *Thermus flavus* (DNAPTfl) as representatives of this class. Polymerase genes from other eubacterial organisms, such as *Thermus thermophilus*, *Thermus sp.*, *Thermotoga maritima*, *Thermosipho africanus* and *Bacillus stearothermophilus* are equally suitable. The DNA polymerases from these thermophilic organisms are capable of surviving and performing at elevated temperatures, and can thus be used in reactions in which temperature is used as a selection against non-specific hybridization of nucleic acid strands.

The restriction sites used for deletion mutagenesis, described below, were chosen for convenience. Different sites situated with similar convenience are available in the *Thermus thermophilus* gene and can be used to make similar constructs with other Type A polymerase genes from related organisms.

A. Creation Of 5' Nuclease Constructs

1. Modified DNAP*Taq* Genes

The first step was to place a modified gene for the *Taq* DNA polymerase on a plasmid under control of an inducible promoter. The modified *Taq* polymerase gene was isolated as follows: The *Taq* DNA polymerase gene was amplified by polymerase chain reaction from genomic DNA from *Thermus aquaticus*, strain YT-1 (Lawyer *et al.*, *supra*), using as primers the oligonucleotides described in SEQ ID NOS:13-14. The resulting fragment of DNA has a recognition sequence for the restriction endonuclease EcoRI at the 5' end of the coding sequence and a BglII sequence at the 3' end. Cleavage with BglII leaves a 5' overhang or "sticky end" that is compatible with the end generated by BamHI. The PCR-amplified DNA was digested with EcoRI and BamHI. The 2512 bp fragment containing the coding region for the polymerase gene was gel purified and then ligated into a plasmid which contains an inducible promoter.

In one embodiment of the invention, the pTTQ18 vector, which contains the hybrid *trp-lac* (*tac*) promoter, was used [M.J.R. Stark, *Gene* 5:255 (1987)] and shown in Fig. 14. The *tac* promoter is under the control of the *E. coli lac* repressor. Repression allows the synthesis of the gene product to be suppressed until the desired level of bacterial growth has been achieved, at which point repression is removed by addition of a specific inducer, isopropyl-β-D-thiogalactopyranoside (IPTG). Such a system allows the expression of foreign proteins that may slow or prevent growth of transformants.

Bacterial promoters, such as *tac*, may not be adequately suppressed when they are present on a multiple copy plasmid. If a highly toxic protein is placed under control of such a promoter, the small amount of expression leaking through can be harmful to the bacteria. In another embodiment of the invention, another option for repressing synthesis of a cloned gene product was used. The non-bacterial promoter, from bacteriophage T7, found in the plasmid vector series pET-3 was used to express the cloned mutant *Taq* polymerase genes [Fig. 15; Studier and Moffatt, *J. Mol. Biol.* 189:113 (1986)]. This promoter initiates transcription only by T7 RNA polymerase. In a suitable strain, such as BL21(DE3)pLYS, the gene for this RNA polymerase is carried on the bacterial genome under control of

the *lac* operator. This arrangement has the advantage that expression of the multiple copy gene (on the plasmid) is completely dependent on the expression of T7 RNA polymerase, which is easily suppressed because it is present in a single copy.

For ligation into the pTTQ18 vector (Fig. 14), the PCR product DNA containing the *Taq* polymerase coding region (mut*Taq*, clone 4B, SEQ ID NO:21) was digested with EcoRI and BglII and this fragment was ligated under standard "sticky end" conditions [Sambrook *et al. Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989)] into the EcoRI and BamHI sites of the plasmid vector pTTQ18. Expression of this construct yields a translational fusion product in which the first two residues of the native protein (Met-Arg) are replaced by three from the vector (Met-Asn-Ser), but the remainder of the natural protein would not change. The construct was transformed into the JM109 strain of *E. coli* and the transformants were plated under incompletely repressing conditions that do not permit growth of bacteria expressing the native protein. These plating conditions allow the isolation of genes containing pre-existing mutations, such as those that result from the infidelity of *Taq* polymerase during the amplification process.

Using this amplification/selection protocol, we isolated a clone (depicted in Fig. 4B) containing a mutated *Taq* polymerase gene (mut*Taq*, clone 4B). The mutant was first detected by its phenotype, in which temperature-stable 5' nuclease activity in a crude cell extract was normal, but polymerization activity was almost absent (approximately less than 1% of wild type *Taq* polymerase activity).

DNA sequence analysis of the recombinant gene showed that it had changes in the polymerase domain resulting in two amino acid substitutions: an A to G change at nucleotide position 1394 causes a Glu to Gly change at amino acid position 465 (numbered according to the natural nucleic and amino acid sequences, SEQ ID NOS:1 and 4) and another A to G change at nucleotide position 2260 causes a Gln to Arg change at amino acid position 754. Because the Gln to Gly mutation is at a nonconserved position and because the Glu to Arg mutation alters an amino acid that is conserved in virtually all of the known Type A polymerases, this latter mutation is most likely the one responsible for curtailing the synthesis

activity of this protein. The nucleotide sequence for the Fig. 4B construct is given in SEQ ID NO:21.

Subsequent derivatives of DNAP*Taq* constructs were made from the mut*Taq* gene, thus, they all bear these amino acid substitutions in addition to their other alterations, unless these particular regions were deleted. These mutated sites are indicated by black boxes at these locations in the diagrams in Fig. 4. All constructs except the genes shown in Figures 4E, F and G were made in the pTTQ18 vector.

The cloning vector used for the genes in Figs. 4E and F was from the commercially available pET-3 series, described above. Though this vector series has only a BamHI site for cloning downstream of the T7 promoter, the series contains variants that allow cloning into any of the three reading frames. For cloning of the PCR product described above, the variant called pET-3c was used (Fig 15). The vector was digested with BamHI, dephosphorylated with calf intestinal phosphatase, and the sticky ends were filled in using the Klenow fragment of DNAPEc1 and dNTPs. The gene for the mutant *Taq* DNAP shown in Fig. 4B (mut*Taq*, clone 4B) was released from pTTQ18 by digestion with EcoRI and Sa1I, and the "sticky ends" were filled in as was done with the vector. The fragment was ligated to the vector under standard blunt-end conditions (Sambrook *et al.*, *Molecular Cloning*, *supra*), the construct was transformed into the BL21(DE3)pLYS strain of *E. coli*, and isolates were screened to identify those that were ligated with the gene in the proper orientation relative to the promoter. This construction yields another translational fusion product, in which the first two amino acids of DNAP*Taq* (Met-Arg) are replaced by 13 from the vector plus two from the PCR primer (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Ile-Asn-Ser) (SEQ ID NO:29).

Our goal was to generate enzymes that lacked the ability to synthesize DNA, but retained the ability to cleave nucleic acids with a 5' nuclease activity. The act of primed, templated synthesis of DNA is actually a coordinated series of events, so it is possible to disable DNA synthesis by disrupting one event while not affecting the others. These steps include, but are not limited to, primer recognition and binding, dNTP binding and catalysis of the inter-nucleotide phosphodiester

bond. Some of the amino acids in the polymerization domain of DNAPEcI have been linked to these functions, but the precise mechanisms are as yet poorly defined.

One way of destroying the polymerizing ability of a DNA polymerase is to delete all or part of the gene segment that encodes that domain for the protein, or to otherwise render the gene incapable of making a complete polymerization domain. Individual mutant enzymes may differ from each other in stability and solubility both inside and outside cells. For instance, in contrast to the 5' nuclease domain of DNAPEcI, which can be released in an active form from the polymerization domain by gentle proteolysis [Setlow and Kornberg, *J. Biol. Chem.* 247:232 (1972)], the *Thermus* nuclease domain, when treated similarly, becomes less soluble and the cleavage activity is often lost.

Using the mutant gene shown in Fig. 4B as starting material, several deletion constructs were created. All cloning technologies were standard (Sambrook *et al.*, *supra*) and are summarized briefly, as follows:

Fig. 4C: The mut*Taq* construct was digested with PstI, which cuts once within the polymerase coding region, as indicated, and cuts immediately downstream of the gene in the multiple cloning site of the vector. After release of the fragment between these two sites, the vector was re-ligated, creating an 894-nucleotide deletion, and bringing into frame a stop codon 40 nucleotides downstream of the junction. The nucleotide sequence of this 5' nuclease (clone 4C) is given in SEQ ID NO:9.

Fig. 4D: The mut*Taq* construct was digested with NheI, which cuts once in the gene at position 2047. The resulting four-nucleotide 5' overhanging ends were filled in, as described above, and the blunt ends were re-ligated. The resulting four-nucleotide insertion changes the reading frame and causes termination of translation ten amino acids downstream of the mutation. The nucleotide sequence of this 5' nuclease (clone 4D) is given in SEQ ID NO:10.

Fig. 4E: The entire mut*Taq* gene was cut from pTTQ18 using EcoRI and SalI and cloned into pET-3c, as described above. This clone was digested with BstXI and XcmI, at unique sites that are situated as shown in Fig. 4E. The DNA was treated with the Klenow fragment of DNAPEc1 and dNTPs, which resulted in

the 3' overhangs of both sites being trimmed to blunt ends. These blunt ends were ligated together, resulting in an out-of-frame deletion of 1540 nucleotides. An in-frame termination codon occurs 18 triplets past the junction site. The nucleotide sequence of this 5' nuclease (clone 4E) is given in SEQ ID NO:11, with the appropriate leader sequence given in SEQ ID NO:30. It is also referred to as Cleavase™ BX.

Fig. 4F: The entire mut*Taq* gene was cut from pTTQ18 using EcoRI and SalI and cloned into pET-3c, as described above. This clone was digested with BstXI and amHI, at unique sites that are situated as shown in the diagram. The DNA was treated with the Klenow fragment of DNAPEc1 and dNTPs, which resulted in the 3' overhang of the BstX I site being trimmed to a blunt end, while the 5' overhang of the Bam HI site was filled in to make a blunt end. These ends were ligated together, resulting in an in-frame deletion of 903 nucleotides. The nucleotide sequence of the 5' nuclease (clone 4F) is given in SEQ ID NO:12. It is also referred to as Cleavase™ BB.

Fig.4G: This polymerase is a variant of that shown in Figure 4E. It was cloned in the plasmid vector pET-21 (Novagen). The non-bacterial promoter from bacteriophage T7, found in this vector, initiates transcription only by T7 RNA polymerase. *See* Studier and Moffatt, *supra*. In a suitable strain, such as (DES)pLYS, the gene for this RNA polymerase is carried on the bacterial genome under control of the *lac* operator. This arrangement has the advantage that expression of the multiple copy gene (on the plasmid) is completely dependent on the expression of T7 RNA polymerase, which is easily suppressed because it is present in a single copy. Because the expression of these mutant genes is under this tightly controlled promoter, potential problems of toxicity of the expressed proteins to the host cells are less of a concern.

The pET-21 vector also features a "His*Tag", a stretch of six consecutive histidine residues that are added on the carboxy terminus of the expressed proteins. The resulting proteins can then be purified in a single step by metal chelation chromatography, using a commerically available (Novagen) column resin with immobilized Ni^{++} ions. The 2.5 ml columns are reusable, and can bind up to 20

mg of the target protein under dative or denaturing (guanidine*HCl or urea) conditions.

E. coli (DES)pLYS cells are transformed with the constructs described above using standard transformation techniques, and used to inoculate a standard growth medium (*e.g.*, Luria-Bertani broth). Production of T7 RNA polymerase is induced during log phase growth by addition of IPTG and incubated for a further 12 to 17 hours. Aliquots of culture are removed both before and after induction and the proteins are examined by SDS-PAGE. Staining with Coomassie Blue allows visualization of the foreign proteins if they account for about 3-5% of the cellular protein and do not co-migrate with any of the major protein bands. Proteins that co-migrate with major host protein must be expressed as more than 10% of the total protein to be seen at this tage of analysis.

Some mutant proteins are sequestered by the cells into inclusion bodies. These are granules that form in the cytoplasm when bacteria are made to express high levels of a foreign protein, and they can be purified from a crude lysate, and analyzed by SDS-PAGE to determine their protein content. If the cloned protein is found in the inclusion bodies, it must be released to assay the cleavage and polymerase activities. Different methods of solubilization may be appropriate for different proteins, and a variety of methods are known. *See e.g.* Builder & Ogez, U.S. Patent No. 4,511,502 (1985); Olson, U.S. Patent No. 4,518,526 (1985); Olson & Pai, U.S. Patent No. 4,511,503 (1985); Jones *et al.*, U.S. Patent No. 4,512,922 (1985), all of which are hereby incorporated by reference.

The solubilized protein is then purified on the Ni^{++} column as described above, following the manufacturers instructions (Novagen). The washed proteins are eluted from the column by a combination of imidazole competitor (1 M) and high salt (0.5 M NaCl), and dialyzed to exchange the buffer and to allow denature proteins to refold. Typical recoveries result in approximately 20 μg of specific protein per ml of starting culture. The DNAP mutant is referred to as Cleavase™ BN and the sequence is given in SEQ ID NO:31.

2. Modified DNAPTfl Gene

The DNA polymerase gene of *Thermus flavus* was isolated from *the "T. flavus"* AT-62 strain obtained from the American Type Tissue Collection (ATCC 33923). This strain has a different restriction map then does the *T. flavus* strain used to generate the sequence published by Akhmetzjanov and Vakhitov, *supra*. The published sequence is listed as SEQ ID NO:2. No sequence data has been published for the DNA polymerase gene from the AT-62 strain of *T. flavus*.

Genomic DNA from *T. flavus* was amplified using the same primers used to amplify the *T. aquaticus* DNA polymerase gene (SEQ ID NOS:13-14). The approximately 2500 base pair PCR fragment was digested with EcoRI and BamHI. The over-hanging ends were made blunt with the Klenow fragment of DNAPEc1 and dNTPs. The resulting approximately 1800 base pair fragment containing the coding region for the N-terminus was ligated into pET-3c, as described above. This construct, clone 5B, is depicted in Fig. 5B. The wild type *T. flavus* DNA polymerase gene is depicted in Fig. 5A. The 5B clone has the same leader amino acids as do the DNAP*Taq* clones 4E and F which were cloned into pET-3c; it is not known precisely where translation termination occurs, but the vector has a strong transcription termination signal immediately downstream of the cloning site.

B. Growth And Induction Of Transformed Cells

Bacterial cells were transformed with the constructs described above using standard transformation techniques and used to inoculate 2 mls of a standard growth medium (*e.g.*, Luria-Bertani broth). The resulting cultures were incubated as appropriate for the particular strain used, and induced if required for a particular expression system. For all of the constructs depicted in Figs. 4 and 5, the cultures were grown to an optical density (at 600nm wavelength) of 0.5 OD.

To induce expression of the cloned genes, the cultures were brought to a final concentration of 0.4 mM IPTG and the incubations were continued for 12 to 17 hours. 50 μl aliquots of each culture were removed both before and after induction and were combined with 20 μl of a standard gel loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequent staining with Coomassie Blue (Sambrook *et al.*, *supra*) allows

visualization of the foreign proteins if they account for about 3-5% of the cellular protein and do not co-migrate with any of the major *E. coli* protein bands. Proteins that do co-migrate with a major host protein must be expressed as more than 10% of the total protein to be seen at this stage of analysis.

C. Heat Lysis And Fractionation

Expressed thermostable proteins, *i.e.*, the 5' nucleases, were isolated by heating crude bacterial cell extracts to cause denaturation and precipitation of the less stable *E. coli* proteins. The precipitated *E. coli* proteins were then, along with other cell debris, removed by centrifugation. 1.7 mls of the culture were pelleted by microcentrifugation at 12,000 to 14,000 rpm for 30 to 60 seconds. After removal of the supernatant, the cells were resuspended in 400 μl of buffer A (50 mM Tris-HC1, pH 7.9, 50 mM dextrose, 1 mM EDTA), re-centrifuged, then resuspended in 80 μl of buffer A with 4mg/ml lysozyme. The cells were incubated at room temperature for 15 minutes, then combined with 80 μl of buffer B (10 mM Tris-HC1, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.5% Tween-20, 0.5% Nonidet-P40).

This mixture was incubated at 75°C for 1 hour to denature and precipitate the host proteins. This cell extract was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the supernatant was transferred to a fresh tube. An aliquot of 0.5 to 1 μl of this supernatant was used directly in each test reaction, and the protein content of the extract was determined by subjecting 7 μl to electrophoretic analysis, as above. The native recombinant *Taq* DNA polymerase [Englke, Anal. Biochem 191:396 (1990)], and the double point mutation protein shown in Fig. 4B are both soluble and active at this point.

The foreign protein may not be detected after the heat treatments due to sequestration of the foreign protein by the cells into inclusion bodies. These are granules that form in the cytoplasm when bacteria are made to express high levels of a foreign protein, and they can be purified from a crude lysate, and analyzed SDS PAGE to determine their protein content. Many methods have been described in the literature, and one approach is described below.

D. Isolation And Solubilization Of Inclusion Bodies

A small culture was grown and induced as described above. A 1.7 ml aliquot was pelleted by brief centrifugation, and the bacterial cells were resuspended in 100 μl of Lysis buffer (50 mM Tris-HC1, pH 8.0, 1 mM EDTA, 100 mM NaCl). 2.5 μl of 20 mM PMSF were added for a final concentration of 0.5 mM, and lysozyme was added to a concentration of 1.0 mg/ml. The cells were incubated at room temperature for 20 minutes, deoxycholic acid was added to 1mg/ml (1 μl of 100 mg/ml solution), and the mixture was further incubated at 37°C for about 15 minutes or until viscous. DNAse I was added to 10 μg/ml and the mixture was incubated at room temperature for about 30 minutes or until it was no longer viscous.

From this mixture the inclusion bodies were collected by centrifugation at 14,000 rpm for 15 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in 100 μl of lysis buffer with 10mM EDTA (pH 8.0) and 0.5% Triton X-100. After 5 minutes at room temperature, the inclusion bodies were pelleted as before, and the supernatant was saved for later analysis. The inclusion bodies were resuspended in 50 μl of distilled water, and 5 μl was combined with SDS gel loading buffer (which dissolves the inclusion bodies) and analyzed electrophoretically, along with an aliquot of the supernatant.

If the cloned protein is found in the inclusion bodies, it may be released to assay the cleavage and polymerase activities and the method of solubilization must be compatible with the particular activity. Different methods of solubilization may be appropriate for different proteins, and a variety of methods are discussed in *Molecular Cloning* (Sambrook *et al.*, *supra*). The following is an adaptation we have used for several of our isolates.

20 μl of the inclusion body-water suspension were pelleted by centrifugation at 14,000 rpm for 4 minutes at room temperature, and the supernatant was discarded. To further wash the inclusion bodies, the pellet was resuspended in 20μl of lysis buffer with 2M urea, and incubated at room temperature for one hour. The washed inclusion bodies were then resuspended in 2 μl of lysis buffer with 8M urea; the solution clarified visibly as the inclusion bodies dissolved. Undissolved

debris was removed by centrifugation at 14,000 rpm for 4 minutes at room temperature, and the extract supernatant was transferred to a fresh tube.

To reduce the urea concentration, the extract was diluted into KH_2PO_4. A fresh tube was prepared containing 180 μl of 50 mM KH_2PO_4, pH 9.5, 1 mM EDTA and 50 mM NaCl. A 2 μl aliquot of the extract was added and vortexed briefly to mix. This step was repeated until all of the extract had been added for a total of 10 additions. The mixture was allowed to sit at room temperature for 15 minutes, during which time some precipitate often forms. Precipitates were removed by centrifugation at 14,000 rpm, for 15 minutes at room temperature, and the supernatant was transferred to a fresh tube. To the 200 μl of protein in the KH_2PO_4 solution, 140-200 μl of saturated $(NH_4)_2SO_4$ were added, so that the resulting mixture was about 41% to 50% saturated $(NH_4)_2SO_4$. The mixture was chilled on ice for 30 minutes to allow the protein to precipitate, and the protein was then collected by centrifugation at 14,000 rpm, for 4 minutes at room temperature. The supernatant was discarded, and the pellet was dissolved in 20 μl Buffer C (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.5% PMSF, 25 mM KCl and 0.5 % each of Tween-20 and Nonidet P 40). The protein solution was centrifuged again for 4 minutes to pellet insoluble materials, and the supernatant was removed to a fresh tube. The protein contents of extracts prepared in this manner were visualized by resolving 1-4 μl by SDS-PAGE; 0.5 to 1 μl of extract was tested in the cleavage and polymerization assays as described.

E. Protein Analysis For Presence Of Nuclease And Synthetic Activity

The 5' nucleases described above and shown in Figs. 4 and 5 were analyzed by the following methods.

1. Structure Specific Nuclease Assay

A candidate modified polymerase is tested for 5' nuclease activity by examining its ability to catalyze structure-specific cleavages. By the term "cleavage structure" as used herein, is meant a nucleic acid structure which is a substrate for cleavage by the 5' nuclease activity of a DNAP.

The polymerase is exposed to test complexes that have the structures shown in Fig. 16. Testing for 5' nuclease activity involves three reactions: 1) a primer-directed cleavage (Fig. 16B) is performed because it is relatively insensitive to variations in the salt concentration of the reaction and can, therefore, be performed in whatever solute conditions the modified enzyme requires for activity; this is generally the same conditions preferred by unmodified polymerases; 2) a similar primer-directed cleavage is performed in a buffer which permits primer-independent cleavage, *i.e.*, a low salt buffer, to demonstrate that the enzyme is viable under these conditions; and 3) a primer-independent cleavage (Fig. 16A) is performed in the same low salt buffer.

The bifurcated duplex is formed between a substrate strand and a template strand as shown in Fig. 16. By the term "substrate strand" as used herein, is meant that strand of nucleic acid in which the cleavage mediated by the 5' nuclease activity occurs. The substrate strand is always depicted as the top strand in the bifurcated complex which serves as a substrate for 5' nuclease cleavage (Fig. 16). By the term "template strand" as used herein, is meant the strand of nucleic acid which is at least partially complementary to the substrate strand and which anneals to the substrate strand to form the cleavage structure. The template strand is always depicted as the bottom strand of the bifurcated cleavage structure (Fig. 16). If a primer (a short oligonucleotide of 19 to 30 nucleotides in length) is added to the complex, as when primer-dependent cleavage is to be tested, it is designed to anneal to the 3' arm of the template strand (Fig. 16B). Such a primer would be extended along the template strand if the polymerase used in the reaction has synthetic activity.

The cleavage structure may be made as a single hairpin molecule, with the 3' end of the target and the 5' end of the pilot joined as a loop as shown in Fig. 16E. A primer oligonucleotide complementary to the 3' arm is also required for these tests so that the enzyme's sensitivity to the presence of a primer may be tested.

Nucleic acids to be used to form test cleavage structures can be chemically synthesized, or can be generated by standard recombinant DNA techniques. By the latter method, the hairpin portion of the molecule can be created by inserting into a

cloning vector duplicate copies of a short DNA segment, adjacent to each other but in opposing orientation. The double-stranded fragment encompassing this inverted repeat, and including enough flanking sequence to give short (about 20 nucleotides) unpaired 5' and 3' arms, can then be released from the vector by restriction enzyme digestion, or by PCR performed with an enzyme lacking a 5' exonuclease (*e.g.*, the Stoffel fragment of Amplitaq™ DNA polymerase, Vent™ DNA polymerase).

The test DNA can be labeled on either end, or internally, with either a radioisotope, or with a non-isotopic tag. Whether the hairpin DNA is a synthetic single strand or a cloned double strand, the DNA is heated prior to use to melt all duplexes. When cooled on ice, the structure depicted in Fig. 16E is formed, and is stable for sufficient time to perform these assays.

To test for primer-directed cleavage (Reaction 1), a detectable quantity of the test molecule (typically 1-100 fmol of ^{32}P-labeled hairpin molecule) and a 10 to 100-fold molar excess of primer are placed in a buffer known to be compatible with the test enzyme. For Reaction 2, where primer-directed cleavage is performed under condition which allow primer-independent cleavage, the same quantities of molecules are placed in a solution that is the same as the buffer used in Reaction 1 regarding pH, enzyme stabilizers (*e.g.*, bovine serum albumin, nonionic detergents, gelatin) and reducing agents (*e.g.*, dithiothreitol, 2-mercaptoethanol) but that replaces any monovalent cation salt with 20 mM KCl; 20 mM KCl is the demonstrated optimum for primer-independent cleavage. Buffers for enzymes, such as DNAPEc1, that usually operate in the absence of salt are not supplemented to achieve this concentration. To test for primer-independent cleavage (Reaction 3) the same quantity of the test molecule, but no primer, are combined under the same buffer conditions used for Reaction 2.

All three test reactions are then exposed to enough of the enzyme that the molar ratio of enzyme to test complex is approximately 1:1. The reactions are incubated at a range of temperatures up to, but not exceeding, the temperature allowed by either the enzyme stability or the complex stability, whichever is lower, up to 80°C for enzymes from thermophiles, for a time sufficient to allow cleavage (10 to 60 minutes). The products of Reactions 1, 2 and 3 are resolved by

denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography or by a comparable method appropriate to the labeling system used. Additional labeling systems include chemiluminescence detection, silver or other stains, blotting and probing and the like. The presence of cleavage products is indicated by the presence of molecules which migrate at a lower molecular weight than does the uncleaved test structure. These cleavage products indicate that the candidate polymerase has structure-specific 5' nuclease activity.

To determine whether a modified DNA polymerase has substantially the same 5' nuclease activity as that of the native DNA polymerase, the results of the above-described tests are compared with the results obtained from these tests performed with the native DNA polymerase. By "substantially the same 5' nuclease activity" we mean that the modified polymerase and the native polymerase will both cleave test molecules in the same manner. It is not necessary that the modified polymerase cleave at the same rate as the native DNA polymerase.

Some enzymes or enzyme preparations may have other associated or contaminating activities that may be functional under the cleavage conditions described above and that may interfere with 5' nuclease detection. Reaction conditions can be modified in consideration of these other activities, to avoid destruction of the substrate, or other masking of the 5' nuclease cleavage and its products. For example, the DNA polymerase I of *E. coli* (Pol I), in addition to its polymerase and 5' nuclease activities, has a 3' exonuclease that can degrade DNA in a 3' to 5' direction. Consequently, when the molecule in Fig. 16E is exposed to this polymerase under the conditions described above, the 3' exonuclease quickly removes the unpaired 3' arm, destroying the bifurcated structure required of a substrate for the 5' exonuclease cleavage and no cleavage is detected. The true ability of Pol I to cleave the structure can be revealed if the 3' exonuclease is inhibited by a change of conditions (*e.g.*, pH), mutation, or by addition of a competitor for the activity. Addition of 500 pmoles of a single-stranded competitor oligonucleotide, unrelated to the Fig. 16E structure, to the cleavage reaction with Pol I effectively inhibits the digestion of the 3' arm of the Fig. 16E structure without interfering with the 5' exonuclease release of the 5' arm. The

concentration of the competitor is not critical, but should be high enough to occupy the 3' exonuclease for the duration of the reaction.

Similar destruction of the test molecule may be caused by contaminants in the candidate polymerase preparation. Several sets of the structure specific nuclease reactions may be performed to determine the purity of the candidate nuclease and to find the window between under and over exposure of the test molecule to the polymerase preparation being investigated.

The above described modified polymerases were tested for 5' nuclease activity as follows: Reaction 1 was performed in a buffer of 10 mM Tris-Cl, pH 8.5 at 20°C, 1.5 mM $MgCl_2$ and 50 mM KCl and in Reaction 2 the KCl concentration was reduced to 20 mM. In Reactions 1 and 2, 10 fmoles of the test substrate molecule shown in Fig. 16E were combined with 1 pmole of the indicated primer and 0.5 to 1.0 µl of extract containing the modified polymerase (prepared as described above). This mixture was then incubated for 10 minutes at 55°C. For all of the mutant polymerases tested these conditions were sufficient to give complete cleavage. When the molecule shown in Fig. 16E was labeled at the 5' end, the released 5' fragment, 25 nucleotides long, was conveniently resolved on a 20% polyacrylamide gel (19:1 cross-linked) with 7 M urea in a buffer containing 45 mM Tris-borate pH 8.3, 1.4 mM EDTA. Clones 4C-F and 5B exhibited structure-specific cleavage comparable to that of the unmodified DNA polymerase. Additionally, clones 4E, 4F and 4G have the added ability to cleave DNA in the absence of a 3' arm as discussed above. Representative cleavage reactions are shown in Figure 17.

For the reactions shown in Fig. 17, the mutant polymerase clones 4E (*Taq* mutant) and 5B (Tfl mutant) were examined for their ability to cleave the hairpin substrate molecule shown in Fig. 16E. The substrate molecule was labeled at the 5' terminus with ^{32}P. 10 fmoles of heat-denatured, end-labeled substrate DNA and 0.5 units of DNAP*Taq* (lane 1) or 0.5 µl of 4e or 5b extract (Fig. 17, lanes 2-7, extract was prepared as described above) were mixed together in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$. The final reaction volume was 10 µl. Reactions shown in lanes 4 and 7 contain in addition 50 µM of each dNTP. Reactions shown in lanes 3, 4, 6 and 7 contain 0.2 µM of

the primer oligonucleotide (complementary to the 3' arm of the substrate and shown in Fig. 16E). Reactions were incubated at 55° C for 4 minutes. Reactions were stopped by the addition of 8 µl of 95% formamide containing 20 mM EDTA and 0.05% marker dyes per 10 µl reaction volume. Samples were then applied to 12% denaturing acrylamide gels. Following electrophoresis, the gels were audoradiographed. Fig. 17 shows that clones 4E and 5B exhibit cleavage activity similar to that of the native DNAP*Taq*. Note that some cleavage occurs in these reactions in the absence of the primer. When long hairpin structure, such as the one used here (Fig. 16E), are used in cleavage reactions performed in buffers containing 50 mM KCl a low level of primer-independent cleavage is seen. Higher concentrations of KCl suppress, but do not elminate, this primer-independent cleavage under these conditions.

2. Assay For Synthetic Activity

The ability of the modified enzyme or proteolytic fragments is assayed by adding the modified enzyme to an assay system in which a primer is annealed to a template and DNA synthesis is catalyzed by the added enzyme. Many standard laboratory techniques employ such an assay. For example, nick translation and enzymatic sequencing involve extension of a primer along a DNA template by a polymerase molecule.

In a preferred assay for determining the synthetic activity of a modified enzyme an oligonucleotide primer is annealed to a single-stranded DNA template, *e.g.*, bacteriophage M13 DNA, and the primer/template duplex is incubated in the presence of the modified polymerase in question, deoxynucleoside triphosphates (dNTPs) and the buffer and salts known to be appropriate for the unmodified or native enzyme. Detection of either primer extension (by denaturing gel electrophoresis) or dNTP incorporation (by acid precipitation or chromatography) is indicative of an active polymerase. A label, either isotopic or non-isotopic, is preferably included on either the primer or as a dNTP to facilitate detection of polymerization products. Synthetic activity is quantified as the amount of free nucleotide incorporated into the growing DNA chain and is expressed as amount incorporated per unit of time under specific reaction conditions.

Representative results of an assay for synthetic activity is shown in Fig. 18. The synthetic activity of the mutant DNAP*Taq* clones 4B-F was tested as follows: A master mixture of the following buffer was made: 1.2X PCR buffer (1X PCR buffer contains 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl, ph 8.5 and 0.05% each Tween 20 and Nonidet P40), 50 μM each of dGTP, dATP and dTTP, 5 μM dCTP and 0.125 μM α-^{32}P-dCTP at 600 Ci/mmol. Before adjusting this mixture to its final volume, it was divided into two equal aliquots. One received distilled water up to a volume of 50 μl to give the concentrations above. The other received 5 μg of single-stranded M13mp18 DNA (approximately 2.5 pmol or 0.05 μM final concentration) and 250 pmol of M13 sequencing primer (5 μM final concentration) and distilled water to a final volume of 50 μl. Each cocktail was warmed to 75°C for 5 minutes and then cooled to room temperature. This allowed the primers to anneal to the DNA in the DNA-containing mixtures.

For each assay, 4 μl of the cocktail with the DNA was combined with 1 μl of the mutant polymerase, prepared as described, or 1 unit of DNAP*Taq* (Perkin Elmer) in 1 μl of dH_2O. A "no DNA" control was done in the presence of the DNAP*Taq* (Fig. 18, lane 1), and a "no enzyme" control was done using water in place of the enzyme (lane 2). Each reaction was mixed, then incubated at room temperature (approx. 22°C) for 5 minutes, then at 55°C for 2 minutes, then at 72°C for 2 minutes. This step incubation was done to detect polymerization in any mutants that might have optimal temperatures lower than 72°C. After the final incubation, the tubes were spun briefly to collect any condensation and were placed on ice. One μl of each reaction was spotted at an origin 1.5 cm from the bottom edge of a polyethyleneimine (PEI) cellulose thin layer chromatography plate and allowed to dry. The chromatography plate was run in 0.75 M NaH_2PO_4, pH 3.5, until the buffer front had run approximately 9 cm from the origin. The plate was dried, wrapped in plastic wrap, marked with luminescent ink, and exposed to X-ray film. Incorporation was detected as counts that stuck where originally spotted, while the unincorporated nucleotides were carried by the salt solution from the origin.

Comparison of the locations of the counts with the two control lanes confirmed the lack of polymerization activity in the mutant preparations. Among

the modified DNAP*Taq* clones, only clone 4B retains any residual synthetic activity as shown in Fig. 18.

EXAMPLE 3

5' Nucleases Derived From Thermostable DNA Polymerases Can Cleave Short Hairpin Structures With Specificity

The ability of the 5' nucleases to cleave hairpin structures to generate a cleaved hairpin structure suitable as a detection molecule was examined. The structure and sequence of the hairpin test molecule is shown in Fig. 19A (SEQ ID NO:15). The oligonucleotide (labeled "primer" in Fig. 19A, SEQ ID NO:22) is shown annealed to its complementary sequence on the 3' arm of the hairpin test molecule. The hairpin test molecule was single-end labeled with ^{32}P using a labeled T7 promoter primer in a polymerase chain reaction. The label is present on the 5' arm of the hairpin test molecule and is represented by the star in Fig. 19A.

The cleavage reaction was performed by adding 10 fmoles of heat-denatured, end-labeled hairpin test molecule, 0.2uM of the primer oligonucleotide (complementary to the 3' arm of the hairpin), 50 μM of each dNTP and 0.5 units of DNAP*Taq* (Perkin Elmer) or 0.5 μl of extract containing a 5' nuclease (prepared as described above) in a total volume of 10 μl in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$. Reactions shown in lanes 3, 5 and 7 were run in the absence of dNTPs.

Reactions were incubated at 55° C for 4 minutes. Reactions were stopped at 55° C by the addition of 8 μl of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 μl reaction volume. Samples were not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM Tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

Fig. 19B shows that altered polymerases lacking any detectable synthetic activity cleave a hairpin structure when an oligonucleotide is annealed to the single-stranded 3' arm of the hairpin to yield a single species of cleaved product (Fig.

19B, lanes 3 and 4). 5' nucleases, such as clone 4D, shown in lanes 3 and 4, produce a single cleaved product even in the presence of dNTPs. 5' nucleases which retain a residual amount of synthetic activity (less than 1% of wild type activity) produce multiple cleavage products as the polymerase can extend the oligonucleotide annealed to the 3' arm of the hairpin thereby moving the site of cleavage (clone 4B, lanes 5 and 6). Native DNA*Taq* produces even more species of cleavage products than do mutant polymerases retaining residual synthetic activity and additionally converts the hairpin structure to a double-stranded form in the presence of dNTPs due to the high level of synthetic activity in the native polymerase (Fig. 19B, lane 8).

EXAMPLE 4

Test Of The Trigger/Detection Assay

To test the ability of an oligonucleotide of the type released in the trigger reaction of the trigger/detection assay to be detected in the detection reaction of the assay, the two hairpin structures shown in Fig. 20A were synthesized using standard techniques. The two hairpins are termed the A-hairpin (SEQ ID NO:23) and the T-hairpin (SEQ ID NO:24). The predicted sites of cleavage in the presence of the appropriate annealed primers are indicated by the arrows. The A- and T-hairpins were designed to prevent intra-strand mis-folding by omitting most of the T residues in the A-hairpin and omitting most of the A residues in the T-hairpin. To avoid mis-priming and slippage, the hairpins were designed with local variations in the sequence motifs (*e.g.*, spacing T residues one or two nucleotides apart or in pairs). The A- and T-hairpins can be annealed together to form a duplex which has appropriate ends for directional cloning in pUC-type vectors; restriction sites are located in the loop regions of the duplex and can be used to elongate the stem regions if desired.

The sequence of the test trigger oligonucleotide is shown in Fig. 20B; this oligonucleotide is termed the alpha primer (SEQ ID NO:25). The alpha primer is complementary to the 3' arm of the T-hairpin as shown in Fig. 20A. When the alpha primer is annealed to the T-hairpin, a cleavage structure is formed that is

recognized by thermostable DNA polymerases. Cleavage of the T-hairpin liberates the 5' single-stranded arm of the T-hairpin, generating the tau primer (SEQ ID NO:26) and a cleaved T-hairpin (Fig. 20B; SEQ ID NO:27). The tau primer is complementary to the 3' arm of the A-hairpin as shown in Fig. 20A. Annealing of the tau primer to the A-hairpin generates another cleavage structure; cleavage of this second cleavage structure liberates the 5' single-stranded arm of the A-hairpin, generating another molecule of the alpha primer which then is annealed to another molecule of the T-hairpin. Thermocycling releases the primers so they can function in additional cleavage reactions. Multiple cycles of annealing and cleavage are carried out. The products of the cleavage reactions are primers and the shortened hairpin structures shown in Fig. 20C. The shortened or cleaved hairpin structures may be resolved from the uncleaved hairpins by electrophoresis on denaturing acrylamide gels.

The annealing and cleavage reactions are carried as follows: In a 50 µl reaction volume containing 10 mM Tris-Cl, pH 8.5, 1.0 $MgCl_2$, 75 mM KCl, 1 pmole of A-hairpin, 1 pmole T-hairpin, the alpha primer is added at equimolar amount relative to the hairpin structures (1 pmole) or at dilutions ranging from 10- to 10^6-fold and 0.5 µl of extract containing a 5' nuclease (prepared as described above) are added. The predicted melting temperature for the alpha or trigger primer is 60°C in the above buffer. Annealing is performed just below this predicted melting temperature at 55°C. Using a Perkin Elmer DNA Thermal Cycler, the reactions are annealed at 55°C for 30 seconds. The temperature is then increased slowly over a five minute period to 72°C to allow for cleavage. After cleavage, the reactions are rapidly brought to 55°C (1°C per second) to allow another cycle of annealing to occur. A range of cycles are performed (20, 40 and 60 cycles) and the reaction products are analyzed at each of these number of cycles. The number of cycles which indicates that the accumulation of cleaved hairpin products has not reached a plateau is then used for subsequent determinations when it is desirable to obtain a quantitative result.

Following the desired number of cycles, the reactions are stopped at 55°C by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 µl reaction volume. Samples are not heated before loading onto

denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

The hairpin molecules may be attached to separate solid support molecules, such as agarose, styrene or magnetic beads, via the 3' end of each hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead if so desired. The advantage of attaching the hairpins to a solid support is that this prevents the hybridization of the A- and T-hairpins to one another during the cycles of melting and annealing. The A- and T-hairpins are complementary to one another (as shown in Fig. 20D) and if allowed to anneal to one another over their entire lengths this would reduce the amount of hairpins available for hybridization to the alpha and tau primers during the detection reaction.

The 5' nucleases of the present invention are used in this assay because they lack significant synthetic activity. The lack of synthetic activity results in the production of a single cleaved hairpin product (as shown in Fig. 19B, lane 4). Multiple cleavage products may be generated by 1) the presence of interfering synthetic activity (*see* Fig. 19B, lanes 6 and 8) or 2) the presence of primer-independent cleavage in the reaction. The presence of primer-independent cleavage is detected in the trigger/detection assay by the presence of different sized products at the fork of the cleavage structure. Primer-independent cleavage can be dampened or repressed, when present, by the use of uncleavable nucleotides in the fork region of the hairpin molecule. For example, thiolated nucleotides can be used to replace several nucleotides at the fork region to prevent primer-independent cleavage.

EXAMPLE 5

Cleavage Of Linear Nucleic Acid Substrates

From the above, it should be clear that native (*i.e.*, "wild type") thermostable DNA polymerases are capable of cleaving hairpin structures in a specific manner and that this discovery can be applied with success to a detection assay. In this example, the mutant DNAPs of the present invention are tested

against three different cleavage structures shown in Figure 22A. Structure 1 in Figure 22A is simply single stranded 206-mer (the preparation and sequence information for which was discussed above). Structures 2 and 3 are duplexes; structure 2 is the same hairpin structure as shown in Figure 12A (bottom), while structure 3 has the hairpin portion of stucture 2 removed.

The cleavage reactions comprised 0.01 pmoles of the resulting substrate DNA, and 1 pmole of pilot oligonucleotide in a total volume of 10 µl of 10 mM Tris-Cl, pH 8.3, 100 mM KCl, 1 mM $MgCl_2$. Reactions were incubated for 30 minutes at 55°C, and stopped by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% polyacrylamide gel (19:1 cross link), with 7M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA.

The results were visualized by autoradiography and are shown in Figure 22B with the enzymes indicated as follows: I is native *Taq* DNAP; II is native *Tfl* DNAP; III is Cleavase™ BX shown in Figure 4E; IV is Cleavase™ BB shown in Figure 4F; V is the mutant shown in Figure 5B; and VI is Cleavase™ BN shown in Figure 4G.

Structure 2 was used to "normalize" the comparison. For example, it was found that it took 50 ng of *Taq* DNAP and 300 ng of Cleavase™ BN to give similar amounts of cleavage of Structure 2 in thirty (30) minutes. Under these conditions native *Taq* DNAP is unable to cleave Structure 3 to any significant degree. Native *Tfl* DNAP cleaves Structure 3 in a manner that creates multiple products.

By contrast, all of the mutants tested cleave the linear duplex of Structure 3. This finding indicates that this characteristic of the mutant DNA polymerases is consistent of thermostable polymerases across thermophilic species.

The finding described herein that the mutant DNA polymerases of the present invention are capable of cleaving linear duplex structures allows for application to a more straightforward assay design (Figure 1A). Figure 23 provides a more detailed schematic corresponding to the assay design of Figure 1A.

The two 43-mers depicted in Figure 23 were synthesized by standard methods. Each included a fluorescein on the 5'end for detection purposes and a

biotin on the 3'end to allow attachment to streptavidin coated paramagnetic particles (the biotin-avidin attachment is indicated by "~~").

Before the trityl groups were removed, the oligos were purified by HPLC to remove truncated by-products of the synthesis reaction. Aliquots of each 43-mer were bound to M-280 Dynabeads (Dynal) at a density of 100 pmoles per mg of beads. Two (2) mgs of beads (200 µl) were washed twice in 1X wash/bind buffer (1 M NaCl, 5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA) with 0.1% BSA, 200 µl per wash. The beads were magnetically sedimented between washes to allow supernatant removal. After the second wash, the beads were resuspended in 200 µl of 2X wash/bind buffer (2 M Na Cl, 10 mM Tris-Cl, pH 7.5 with 1 mM EDTA), and divided into two 100 µl aliquots. Each aliquot received 1 µl of a 100 µM solution of one of the two oligonucleotides. After mixing, the beads were incubated at room temperature for 60 minutes with occasional gentle mixing. The beads were then sedimented and analysis of the supernatants showed only trace amounts of unbound oligonucleotide, indicating successful binding. Each aliquot of beads was washed three times, 100 µl per wash, with 1X wash/bind buffer, then twice in a buffer of 10 mM Tris-Cl, pH 8.3 and 75 mM KCl. The beads were resuspended in a final volume of 100 µl of the Tris/KCl, for a concentration of 1 pmole of oligo bound to 10 µg of beads per µl of suspension. The beads were stored at 4°C between uses.

The types of beads correspond to Figure 1A. That is to say, type 2 beads contain the oligo (SEQ ID NO:33) comprising the complementary sequence (SEQ ID NO:34) for the alpha signal oligo (SEQ ID NO:35) as well as the beta signal oligo (SEQ ID NO:36) which when liberated is a 24-mer. This oligo has no "As" and is "T" rich. Type 3 beads contain the oligo (SEQ ID NO:37) comprising the complementary sequence (SEQ ID NO:38) for the beta signal oligo (SEQ ID NO:39) as well as the alpha signal oligo (SEQ ID NO:35) which when liberated is a 20-mer. This oligo has no "Ts" and is "A" rich.

Cleavage reactions comprised 1 µl of the indicated beads, 10 pmoles of unlabelled alpha signal oligo as "pilot" (if indicated) and 500 ng of Cleavase™ BN in 20 µl of 75 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$ and 10 µM CTAB. All components except the enzyme were assembled, overlaid with light

mineral oil and warmed to 53°C. The reactions were initiated by the addition of prewarmed enzyme and incubated at that temperature for 30 minutes. Reactions were stopped at temperature by the addition of 16 µl of 95% formamide with 20 mM EDTA and 0.05% each of bromophenol blue and xylene cyanol. This addition stops the enzyme activity and, upon heating, disrupts the biotin-avidin link, releasing the majority (greater than 95%) of the oligos from the beads. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% polyacrylamide gel (19:1 cross link), with 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. Results were visualized by contact transfer of the resolved DNA to positively charged nylon membrane and probing of the blocked membrane with an anti-fluorescein antibody conjugated to alkaline phosphatase. After washing, the signal was developed by incubating the membrane in Western Blue (Promega) which deposits a purple precipitate where the antibody is bound.

Figure 24 shows the propagation of cleavage of the linear duplex nucleic acid structures of Figure 23 by the DNAP mutants of the present invention. The two center lanes contain both types of beads. As noted above, the beta signal oligo (SEQ ID NO:36) when liberated is a 24-mer and the alpha signal oligo (SEQ ID NO:35) when liberated is a 20-mer. The formation of the two lower bands corresponding to the 24-mer and 20-mer is clearly dependent on "pilot".

EXAMPLE 6

5' Exonucleolytic Cleavage ("Nibbling") By Thermostable DNAPs

It has been found that thermostable DNAPs, including those of the present invention, have a true 5' exonuclease capable of nibbling the 5' end of a linear duplex nucleic acid structures. In this example, the 206 base pair DNA duplex substrate is again employed (see above). In this case, it was produced by the use of one ^{32}P-labeled primer and one unlabeled primer in a polymerase chain reaction. The cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled substrate DNA (with the unlabeled strand also present), 5 pmoles of pilot oligonucleotide (see pilot oligos in Figure 12A) and 0.5 units of DNAPTaq or 0.5

μ of Cleavase™ BB in the *E. coli* extract (see above), in a total volume of 10 μl of 10 mM Tris•Cl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$.

Reactions were initiated at 65°C by the addition of pre-warmed enzyme, then shifted to the final incubation temperature for 30 minutes. The results are shown in Figure 25A. Samples in lanes 1-4 are the results with native *Taq* DNAP, while lanes 5-8 shown the results with Cleavase™ BB. The reactions for lanes 1, 2, 5, and 6 were performed at 65°C and reactions for lanes 3, 4, 7, and 8 were performed at 50°C and all were stopped at temperature by the addition of 8 μl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA. The expected product in reactions 1, 2, 5, and 6 is 85 nucleotides long; in reactions 3 and 7, the expected product is 27 nucleotides long. Reactions 4 and 8 were performed without pilot, and should remain at 206 nucleotides. The faint band seen at 24 nucleotides is residual end-labeled primer from the PCR.

The surprising result is that Cleavase™ BB under these conditions causes all of the label to appear in a very small species, suggesting the possibility that the enzyme completely hydrolyzed the substrate. To determine the composition of the fastest-migrating band seen in lanes 5-8 (reactions performed with the deletion mutant), samples of the 206 base pair duplex were treated with either T7 gene 6 exonuclease (USB) or with calf intestine alkaline phosphatase (Promega), according to manufacturers' instructions, to produce either labeled mononucleotide (lane a of Figure 25B) or free ^{32}P-labeled inorganic phosphate (lane b of Figure 25B), respectively. These products, along with the products seen in lane 7 of panel A were resolved by brief electrophoresis through a 20% acrylamide gel (19:1 cross-link), with 7 M urea, in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA. Cleavase™ BB is thus capable of converting the substrate to mononucleotides.

EXAMPLE 7

Nibbling Is Duplex Dependent

The nibbling by Cleavase™ BB is duplex dependent. In this example, internally labeled, single strands of the 206-mer were produced by 15 cycles of primer extension incorporating α-^{32}P labeled dCTP combined with all four unlabeled dNTPs, using an unlabeled 206-bp fragment as a template. Single and double stranded products were resolved by electrophoresis through a non-denaturing 6% polyacrylamide gel (29:1 cross-link) in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA, visualized by autoradiography, excised from the gel, eluted by passive diffusion, and concentrated by ethanol precipitation.

The cleavage reactions comprised 0.04 pmoles of substrate DNA, and 2 µl of Cleavase™ BB (in an *E. coli* extract as described above) in a total volume of 40 µl of 10 mM Tris•Cl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$. Reactions were initiated by the addition of pre-warmed enzyme; 10 µl aliquots were removed at 5, 10, 20, and 30 minutes, and transferred to prepared tubes containing 8 µl of 95% formamide with 30 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA. Results were visualized by autoradiography as shown in Figure 26. Clearly, the cleavage by Cleavase™ BB depends on a duplex structure; no cleavage of the single strand structure is detected whereas cleavage of the 206-mer duplex is complete.

EXAMPLE 8

Nibbling Can Be Target Directed

The nibbling activity of the DNAPs of the present invention can be employed with success in a detection assay. One embodiment of such an assay is shown in Figure 27. In this assay, a labelled oligo is employed that is specific for a target sequence. The oligo is in excess of the target so that hybridization is rapid. In this embodiment, the oligo contains two fluorescein labels whose

proximity on the oligo causes their emmision to be quenched. When the DNAP is permitted to nibble the oligo the labels separate and are detectable. The shortened duplex is destabilized and disassociates. Importantly, the target is now free to react with an intact labelled oligo. The reaction can continue until the desired level of detection is achieved. An analogous, although different, type of cycling assay has been described employing lambda exonuclease. *See* C.G. Copley and C. Boot, *BioTechniques* 13:888 (1992).

The success of such an assay depends on specificity. In other words, the oligo must hybridize to the specific target. It is also preferred that the assay be sensitive; the oligo ideally should be able to detect small amounts of target. Figure 28A shows a 5'-end ^{32}P-labelled primer bound to a plasmid target sequence. In this case, the plasmid was pUC19 (commercially available) which was heat denatured by boiling two (2) minutes and then quick chilling. The primer is a 21-mer (SEQ ID NO:39). The enzyme employed was Cleavase™ BX (a dilution equivalent to 5 x 10^{-3} µl extract) in 100 mM KCl, 10 mM Tris-Cl, pH 8.3, 2 mM $MnCl_2$. The reaction was performed at 55°C for sixteen (16) hours with or without genomic background DNA (from chicken blood). The reaction was stopped by the addition of 8 µl of 95% formamide with 20 mM EDTA and marker dyes.

The products of the reaction were resolved by PAGE (10% polyacrylamide, 19:1 cross link, 1 x TBE) as seen in Figure 28B. Lane "M" contains the labelled 21-mer. Lanes 1-3 contain no specific target, although Lanes 2 and 3 contain 100 ng and 200 ng of genomic DNA, respectively. Lanes 4, 5 and 6 all contain specific target with either 0 ng, 100 ng or 200 ng of genomic DNA, respectively. It is clear that conversion to mononucleotides occurs in Lanes 4, 5 and 6 regardless of the presence or amount of background DNA. Thus, the nibbling can be target directed and specific.

EXAMPLE 9

Cleavase Purification

As noted above, expressed thermostable proteins, *i.e.*, the 5' nucleases, were isolated by crude bacterial cell extracts. The precipitated *E. coli* proteins were then, along with other cell debris, removed by centrifugation. In this example, cells expressing the BN clone were cultured and collected (500 grams). For each gram (wet weight) of *E. coli*, 3ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100μM NaCl) was added. The cells were lysed with 200 ug/ml lysozyme at room temperature for 20 minutes. Thereafter deoxycholic acid was added to make a 0.2% final concentration and the mixture was incubated 15 minutes at room temperature.

The lysate was sonicated for approximately 6-8 minutes at 0°C. The precipitate was removed by centriguation (39,000g for 20 minutes). Polyethyleneimine was added (0.5%) to the supernatant and the mixture was incubated on ice for 15 minutes.

The mixture was centrifuged (5,000g for 15 minutes) and the supernatant was retained. This was heated for 30 minutes at 60°C and then centrifuged again (5,000g for 15 minutes) and the supernatant was again retained.

The supernatant was precipitated with 35% ammonium sulfate at 4°C for 15 minutes. The mixture was then centrifuged (5,000g for 15 minutes) and the supernatant was removed. The precipitate was then dissolved in 0.25 M KCl, 20 Tris pH 7.6, 0.2% Tween and 0.1 EDTA) and then dialyzed against Binding Buffer (8X Binding Buffer comprises: 40mM imidazole, 4M NaCl, 160 mM Tris-HCl, pH 7.9).

The solubilized protein is then purified on the Ni^{++} column (Novagen). The Binding Buffer is allows to drain to the top of the column bed and load the column with the prepared extract. A flow rate of about 10 column volumes per hour is optimal for efficient purification. If the flow rate is too fast, more impurities will contaminate the eluted fraction.

The column is washed with 25 ml (10 volumes) of 1X Binding Buffer and then washed with 15 ml (6 volumes) of 1X Wash Buffer (8X Wash Buffer

comprises: 480mM imidazole, 4M NaCl, 160 mM Tris-HCl, pH 7.9). The bound protein was eluted with 15ml (6 volumes) of 1X Elute Buffer (4X Elute Buffer comprises: 4mM imidazole, 2M NaCl, 80 mM Tris-HCl, pH 7.9). Protein is then reprecipitated with 35% Ammonium Sulfate as above. The precipitate was then dissolved and dialyzed against: 20 mM Tris, 100 mM KC1, 1mM EDTA). The solution was brought up to 0.1% each of Tween 20 and NP-40 and stored at 4°C.

From the above, it should be clear that the present invention provides novel cleaving enzymes having heretofore undisclosed nuclease activities. The enzymes can be employed with success in target detection assays of various designs. These assays do not require that the sample DNA be amplified prior to detection and therefore offer an improvement in DNA-based detection technology.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: 5' Nucleases Derived From Thermostable DNA Polymerase

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2505 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGGGGGA TGCTGCCCCT CTTTGAGCCC AAGGGCCGGG TCCTCCTGGT GGACGGCCAC 60

CACCTGGCCT ACCGCACCTT CCACGCCCTG AAGGGCCTCA CCACCAGCCG GGGGGAGCCG 120

GTGCAGGCGG TCTACGGCTT CGCCAAGAGC CTCCTCAAGG CCCTCAAGGA GGACGGGGAC 180

GCGGTGATCG TGGTCTTTGA CGCCAAGGCC CCCTCCTTCC GCCACGAGGC CTACGGGGGG 240

TACAAGGCGG GCCGGGCCCC CACGCCGGAG GACTTTCCCC GGCAACTCGC CCTCATCAAG 300

GAGCTGGTGG ACCTCCTGGG GCTGGCGCGC CTCGAGGTCC CGGGCTACGA GGCGGACGAC 360

GTCCTGGCCA GCCTGGCCAA GAAGGCGGAA AAGGAGGGCT ACGAGGTCCG CATCCTCACC 420

GCCGACAAAG ACCTTTACCA GCTCCTTTCC GACCGCATCC ACGTCCTCCA CCCCGAGGGG 480

TACCTCATCA CCCCGGCCTG GCTTTGGGAA AAGTACGGCC TGAGGCCCGA CCAGTGGGCC 540

GACTACCGGG CCCTGACCGG GGACGAGTCC GACAACCTTC CCGGGGTCAA GGGCATCGGG 600

GAGAAGACGG CGAGGAAGCT TCTGGAGGAG TGGGGGAGCC TGGAAGCCCT CCTCAAGAAC 660

CTGGACCGGC TGAAGCCCGC CATCCGGGAG AAGATCCTGG CCCACATGGA CGATCTGAAG 720

CTCTCCTGGG ACCTGGCCAA GGTGCGCACC GACCTGCCCC TGGAGGTGGA CTTCGCCAAA 780

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CCGCCGGAAG GGGCCTTCGT GGGCTTTGTG CTTTCCCGCA AGGAGCCCAT GTGGGCCGAT 960

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GCCCTCAGGG ACCTGAAGGA GGCGCGGGGG CTTCTCGCCA AAGACCTGAG CGTTCTGGCC 1080

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GACCCTTCCA ACACCACCCC CGAGGGGGTG GCCCGGCGCT ACGGCGGGGA GTGGACGGAG 1200

GAGGCGGGGG AGCGGGCCGC CCTTTCCGAG AGGCTCTTCG CCAACCTGTG GGGGAGGCTT 1260

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CCCTTCAACC TCAACTCCCG GGACCAGCTG GAAAGGGTCC TCTTTGACGA GCTAGGGCTT 1500

CCCGCCATCG GCAAGACGGA GAAGACCGGC AAGCGCTCCA CCAGCGCCGC CGTCCTGGAG 1560

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CTGAAGAGCA CCTACATTGA CCCCTTGCCG GACCTCATCC ACCCCAGGAC GGGCCGCCTC 1680

CACACCCGCT TCAACCAGAC GGCCACGGCC ACGGGCAGGC TAAGTAGCTC CGATCCCAAC 1740

CTCCAGAACA TCCCCGTCCG CACCCCGCTT GGGCAGAGGA TCCGCCGGGC CTTCATCGCC 1800

GAGGAGGGGT GGCTATTGGT GGCCCTGGAC TATAGCCAGA TAGAGCTCAG GGTGCTGGCC 1860

CACCTCTCCG GCGACGAGAA CCTGATCCGG GTCTTCCAGG AGGGGCGGGA CATCCACACG 1920

GAGACCGCCA GCTGGATGTT CGGCGTCCCC CGGGAGGCCG TGGACCCCCT GATGCGCCGG 1980

GCGGCCAAGA CCATCAACTT CGGGGTCCTC TACGGCATGT CGGCCCACCG CCTCTCCCAG 2040

GAGCTAGCCA TCCTTACGAG GAGGCCCAGG CCTTCATTGA GCGCTACTTT CAGAGCTTCC 2100

CCAAGGTGCG GGCCTGGATT GAGAAGACCC TGGAGGAGGG CAGGAGGCGG GGGTACGTGG 2160

AGACCCTCTT CGGCCGCCGC CGCTACGTGC CAGACCTAGA GGCCCGGGTG AAGAGCGTGC 2220

GGGAGGCGGC CGAGCGCATG GCCTTCAACA TGCCCGTCCA GGGCACCGCC GCCGACCTCA 2280

TGAAGCTGGC TATGGTGAAG CTCTTCCCCA GGCTGGAGGA AATGGGGGCC AGGATGCTCC 2340

TTCAGGTCCA CGACGAGCTG GTCCTCGAGG CCCCAAAAGA GAGGGCGGAG GCCGTGGCCC 2400

GGCTGGCCAA GGAGGTCATG GAGGGGGTGT ATCCCCTGGC CGTGCCCCTG GAGGTGGAGG 2460

TGGGGATAGG GGAGGACTGG CTCTCCGCCA AGGAGTGATA CCACC 2505

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2496 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGCGATGC TTCCCCTCTT TGAGCCCAAA GGCCGCGTGC TCCTGGTGGA CGGCCACCAC 60

CTGGCCTACC GCACCTTCTT TGCCCTCAAG GGCCTCACCA CCAGCCGCGG CGAACCCGTT 120

CAGGCGGTCT ACGGCTTCGC CAAAAGCCTC CTCAAGGCCC TGAAGGAGGA CGGGGACGTG 180

GTGGTGGTGG TCTTTGACGC CAAGGCCCCC TCCTTCCGCC ACGAGGCCTA CGAGGCCTAC 240

AAGGCGGGCC GGGCCCCCAC CCCGGAGGAC TTTCCCCGGC AGCTGGCCCT CATCAAGGAG 300

TTGGTGGACC TCCTAGGCCT TGTGCGGCTG GAGGTTCCCG GCTTTGAGGC GGACGACGTG 360

CTGGCCACCC TGGCCAAGCG GGCGGAAAAG GAGGGGTACG AGGTGCGCAT CCTCACTGCC 420

GACCGCGACC TCTACCAGCT CCTTTCGGAG CGCATCGCCA TCCTCCACCC TGAGGGGTAC 480

CTGATCACCC CGGCGTGGCT TTACGAGAAG TACGGCCTGC GCCCGGAGCA GTGGGTGGAC 540

TACCGGGCCC TGGCGGGGGA CCCCTCGGAT AACATCCCCG GGGTGAAGGG CATCGGGGAG 600

AAGACCGCCC AGAGGCTCAT CCGCGAGTGG GGGAGCCTGG AAAACCTCTT CCAGCACCTG 660

GACCAGGTGA AGCCCTCCTT GCGGGAGAAG CTCCAGGCGG GCATGGAGGC CCTGGCCCTT 720

TCCCGGAAGC TTTCCCAGGT GCACACTGAC CTGCCCCTGG AGGTGGACTT CGGGAGGCGC 780

CGCACACCCA ACCTGGAGGG TCTGCGGGCT TTTTTGGAGC GGTTGGAGTT TGGAAGCCTC 840

CTCCACGAGT TCGGCCTCCT GGAGGGGCCG AAGGCGGCAG AGGAGGCCCC CTGGCCCCCT 900

CCGGAAGGGG CTTTTTTGGG CTTTTCCTTT TCCCGTCCCG AGCCCATGTG GGCCGAGCTT 960

CTGGCCCTGG CTGGGGCGTG GGAGGGGCGC CTCCATCGGG CACAAGACCC CCTTAGGGGC 1020

CTGAGGGACC TTAAGGGGGT GCGGGGAATC CTGGCCAAGG ACCTGGCGGT TTTGGCCCTG 1080

CGGGAGGGCC TGGACCTCTT CCCAGAGGAC GACCCCATGC TCCTGGCCTA CCTTCTGGAC 1140

CCCTCCAACA CCACCCCTGA GGGGGTGGCC CGGCGTTACG GGGGGGAGTG GACGGAGGAT 1200

GCGGGGGAGA GGGCCCTCCT GGCCGAGCGC CTCTTCCAGA CCCTAAAGGA GCGCCTTAAG 1260

GGAGAAGAAC GCCTGCTTTG GCTTTACGAG GAGGTGGAGA AGCCGCTTTC CCGGGTGTTG 1320

GCCCGGATGG AGGCCACGGG GGTCCGGCTG GACGTGGCCT ACCTCCAGGC CCTCTCCCTG 1380

GAGGTGGAGG CGGAGGTGCG CCAGCTGGAG GAGGAGGTCT TCCGCCTGGC CGGCCACCCC 1440

TTCAACCTCA ACTCCCGCGA CCAGCTGGAG CGGGTGCTCT TTGACGAGCT GGGCCTGCCT 1500

GCCATCGGCA AGACGGAGAA GACGGGGAAA CGCTCCACCA GCGCTGCCGT GCTGGAGGCC 1560

CTGCGAGAGG CCCACCCCAT CGTGGACCGC ATCCTGCAGT ACCGGGAGCT CACCAAGCTC 1620

AAGAACACCT ACATAGACCC CCTGCCCGCC CTGGTCCACC CCAAGACCGG CCGGCTCCAC 1680

ACCCGCTTCA ACCAGACGGC CACCGCCACG GGCAGGCTTT CCAGCTCCGA CCCCAACCTG 1740

CAGAACATCC CCGTGCGCAC CCCTCTGGGC CAGCGCATCC GCCGAGCCTT CGTGGCCGAG 1800

GAGGGCTGGG TGCTGGTGGT CTTGGACTAC AGCCAGATTG AGCTTCGGGT CCTGGCCCAC 1860

CTCTCCGGGG ACGAGAACCT GATCCGGGTC TTTCAGGAGG GGAGGGACAT CCACACCCAG 1920

ACCGCCAGCT GGATGTTCGG CGTTTCCCCC GAAGGGGTAG ACCCTCTGAT GCGCCGGGCG 1980

GCCAAGACCA TCAACTTCGG GGTGCTCTAC GGCATGTCCG CCCACCGCCT CTCCGGGGAG 2040

CTTTCCATCC CCTACGAGGA GGCGGTGGCC TTCATTGAGC GCTACTTCCA GAGCTACCCC 2100

AAGGTGCGGG CCTGGATTGA GGGGACCCTC GAGGAGGGCC GCCGGCGGGG GTATGTGGAG 2160

ACCCTCTTCG GCCGCCGGCG CTATGTGCCC GACCTCAACG CCCGGGTGAA GAGCGTGCGC 2220

GAGGCGGCGG AGCGCATGGC CTTCAACATG CCGGTCCAGG GCACCGCCGC CGACCTCATG 2280

AAGCTGGCCA TGGTGCGGCT TTTCCCCCGG CTTCAGGAAC TGGGGGCGAG GATGCTTTTG 2340

CAGGTGCACG ACGAGCTGGT CCTCGAGGCC CCCAAGGACC GGGCGGAGAG GGTAGCCGCT 2400

TTGGCCAAGG AGGTCATGGA GGGGGTCTGG CCCCTGCAGG TGCCCCTGGA GGTGGAGGTG 2460

GGCCTGGGGG AGGACTGGCT CTCCGCCAAG GAGTAG 2496

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2504 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGAGGCGA TGCTTCCGCT CTTTGAACCC AAAGGCCGGG TCCTCCTGGT GGACGGCCAC 60

CACCTGGCCT ACCGCACCTT CTTCGCCCTG AAGGGCCTCA CCACGAGCCG GGGCGAACCG 120

GTGCAGGCGG TCTACGGCTT CGCCAAGAGC CTCCTCAAGG CCCTGAAGGA GGACGGGTAC 180

AAGGCCGTCT TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGAG 240

GCCTACAAGG CGGGGAGGGC CCCGACCCCC GAGGACTTCC CCCGGCAGCT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGGTTTACC CGCCTCGAGG TCCCCGGCTA CGAGGCGGAC 360

GACGTTCTCG CCACCCTGGC CAAGAAGGCG GAAAAGGAGG GGTACGAGGT GCGCATCCTC 420

ACCGCCGACC GCGACCTCTA CCAACTCGTC TCCGACCGCG TCGCCGTCCT CCACCCCGAG 480

GGCCACCTCA TCACCCCGGA GTGGCTTTGG GAGAAGTACG GCCTCAGGCC GGAGCAGTGG 540

GTGGACTTCC GCGCCCTCGT GGGGGACCCC TCCGACAACC TCCCCGGGGT CAAGGGCATC 600

GGGGAGAAGA CCGCCCTCAA GCTCCTCAAG GAGTGGGGAA GCCTGGAAAA CCTCCTCAAG 660

AACCTGGACC GGGTAAAGCC AGAAAACGTC CGGGAGAAGA TCAAGGCCCA CCTGGAAGAC 720

CTCAGGCTCT CCTTGGAGCT CTCCCGGGTG CGCACCGACC TCCCCCTGGA GGTGGACCTC 780

GCCCAGGGGC GGGAGCCCGA CCGGGAGGGG CTTAGGGCCT TCCTGGAGAG GCTGGAGTTC 840

GGCAGCCTCC TCCACGAGTT CGGCCTCCTG GAGGCCCCCG CCCCCCTGGA GGAGGCCCCC 900

TGGCCCCCGC CGGAAGGGGC CTTCGTGGGC TTCGTCCTCT CCCGCCCCGA GCCCATGTGG 960

GCGGAGCTTA AAGCCCTGGC CGCCTGCAGG GACGGCCGGG TGCACCGGGC AGCAGACCCC 1020

TTGGCGGGGC TAAAGGACCT CAAGGAGGTC CGGGGCCTCC TCGCCAAGGA CCTCGCCGTC 1080

TTGGCCTCGA GGGAGGGGCT AGACCTCGTG CCCGGGGACG ACCCCATGCT CCTCGCCTAC 1140

CTCCTGGACC CCTCCAACAC CACCCCCGAG GGGGTGGCGC GGCGCTACGG GGGGGAGTGG 1200

ACGGAGGACG CCGCCCACCG GGCCCTCCTC TCGGAGAGGC TCCATCGGAA CCTCCTTAAG 1260

CGCCTCGAGG GGGAGGAGAA GCTCCTTTGG CTCTACCACG AGGTGGAAAA GCCCCTCTCC 1320

CGGGTCCTGG CCCACATGGA GGCCACCGGG GTACGGCTGG ACGTGGCCTA CCTTCAGGCC 1380

CTTTCCCTGG AGCTTGCGGA GGAGATCCGC CGCCTCGAGG AGGAGGTCTT CCGCTTGGCG 1440

GGCCACCCCT TCAACCTCAA CTCCCGGGAC CAGCTGGAAA GGGTGCTCTT TGACGAGCTT 1500

AGGCTTCCCG CCTTGGGGAA GACGCAAAAG ACAGGCAAGC GCTCCACCAG CGCCGCGGTG 1560

CTGGAGGCCC TACGGGAGGC CCACCCCATC GTGGAGAAGA TCCTCCAGCA CCGGGAGCTC 1620

ACCAAGCTCA AGAACACCTA CGTGGACCCC CTCCCAAGCC TCGTCCACCC GAGGACGGGC 1680

CGCCTCCACA CCCGCTTCAA CCAGACGGCC ACGGCCACGG GGAGGCTTAG TAGCTCCGAC 1740

CCCAACCTGC AGAACATCCC CGTCCGCACC CCCTTGGGCC AGAGGATCCG CCGGGCCTTC 1800

GTGGCCGAGG CGGGTTGGGC GTTGGTGGCC CTGGACTATA GCCAGATAGA GCTCCGCGTC 1860

CTCGCCCACC TCTCCGGGGA CGAAAACCTG ATCAGGGTCT TCCAGGAGGG GAAGGACATC 1920

CACACCCAGA CCGCAAGCTG GATGTTCGGC GTCCCCCCGG AGGCCGTGGA CCCCCTGATG 1980

CGCCGGGCGG CCAAGACGGT GAACTTCGGC GTCCTCTACG GCATGTCCGC CCATAGGCTC 2040

TCCCAGGAGC TTGCCATCCC CTACGAGGAG GCGGTGGCCT TTATAGAGGC TACTTCCAAA 2100

GCTTCCCCAA GGTGCGGGCC TGGATAGAAA AGACCCTGGA GGAGGGGAGG AAGCGGGGCT 2160

ACGTGGAAAC CCTCTTCGGA AGAAGGCGCT ACGTGCCCGA CCTCAACGCC CGGGTGAAGA 2220

GCGTCAGGGA GGCCGCGGAG CGCATGGCCT TCAACATGCC CGTCCAGGGC ACCGCCGCCG 2280

ACCTCATGAA GCTCGCCATG GTGAAGCTCT TCCCCCGCCT CCGGGAGATG GGGGCCCGCA 2340

TGCTCCTCCA GGTCCACGAC GAGCTCCTCC TGGAGGCCCC CCAAGCGCGG GCCGAGGAGG 2400

TGGCGGCTTT GGCCAAGGAG GCCATGGAGA AGGCCTATCC CCTCGCCGTG CCCCTGGAGG 2460

TGGAGGTGGG GATGGGGGAG GACTGGCTTT CCGCCAAGGG TTAG 2504

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 832 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Gly Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
1 5 10 15

Val Asp Gly His His Leu Ala Tyr Arg Thr Phe His Ala Leu Lys Gly
20 25 30

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
35 40 45

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Ile Val
50 55 60

Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Gly Gly
65 70 75 80

Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu
85 90 95

Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Ala Arg Leu Glu
100 105 110

Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Ser Leu Ala Lys Lys
115 120 125

Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Lys Asp
130 135 140

Leu Tyr Gln Leu Leu Ser Asp Arg Ile His Val Leu His Pro Glu Gly
145 150 155 160

Tyr Leu Ile Thr Pro Ala Trp Leu Trp Glu Lys Tyr Gly Leu Arg Pro
165 170 175

Asp Gln Trp Ala Asp Tyr Arg Ala Leu Thr Gly Asp Glu Ser Asp Asn
180 185 190

Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Arg Lys Leu Leu
195 200 205

Glu Glu Trp Gly Ser Leu Glu Ala Leu Leu Lys Asn Leu Asp Arg Leu
210 215 220

Lys Pro Ala Ile Arg Glu Lys Ile Leu Ala His Met Asp Asp Leu Lys
225 230 235 240

Leu Ser Trp Asp Leu Ala Lys Val Arg Thr Asp Leu Pro Leu Glu Val
245 250 255

Asp Phe Ala Lys Arg Arg Glu Pro Asp Arg Glu Arg Leu Arg Ala Phe
260 265 270

Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu
275 280 285

Glu Ser Pro Lys Ala Leu Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly
290 295 300

Ala Phe Val Gly Phe Val Leu Ser Arg Lys Glu Pro Met Trp Ala Asp
305 310 315 320

Leu Leu Ala Leu Ala Ala Ala Arg Gly Gly Arg Val His Arg Ala Pro
325 330 335

Glu Pro Tyr Lys Ala Leu Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu
340 345 350

Ala Lys Asp Leu Ser Val Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro
355 360 365

Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn
370 375 380

Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu
385 390 395 400

Glu Ala Gly Glu Arg Ala Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu
405 410 415

Trp Gly Arg Leu Glu Gly Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu
420 425 430

Val Glu Arg Pro Leu Ser Ala Val Leu Ala His Met Glu Ala Thr Gly
435 440 445

Val Arg Leu Asp Val Ala Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala
450 455 460

Glu Glu Ile Ala Arg Leu Glu Ala Glu Val Phe Arg Leu Ala Gly His
465 470 475 480

Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp
485 490 495

Glu Leu Gly Leu Pro Ala Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg
500 505 510

Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile
515 520 525

Val Glu Lys Ile Leu Gln Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr
530 535 540

Tyr Ile Asp Pro Leu Pro Asp Leu Ile His Pro Arg Thr Gly Arg Leu
545 550 555 560

His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser
565 570 575

Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln
580 585 590

Arg Ile Arg Arg Ala Phe Ile Ala Glu Glu Gly Trp Leu Leu Val Ala
595 600 605

Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly
610 615 620

Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr
625 630 635 640

Glu Thr Ala Ser Trp Met Phe Gly Val Pro Arg Glu Ala Val Asp Pro
645 650 655

Leu Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly
660 665 670

Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
675 680 685

Ala Gln Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg
690 695 700

Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val
705 710 715 720

Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg
725 730 735

Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro
740 745 750

Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu
755 760 765

Phe Pro Arg Leu Glu Glu Met Gly Ala Arg Met Leu Leu Gln Val His
770 775 780

Asp Glu Leu Val Leu Glu Ala Pro Lys Glu Arg Ala Glu Ala Val Ala
785 790 795 800

Arg Leu Ala Lys Glu Val Met Glu Gly Val Tyr Pro Leu Ala Val Pro
805 810 815

Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu
820 825 830

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 831 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu Val
1 5 10 15

Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly Leu
20 25 30

Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala Lys
35 40 45

Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Val Val Val Val Val
50 55 60

Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu Ala Tyr
65 70 75 80

Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu Ala
85 90 95

Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Val Arg Leu Glu Val
100 105 110

Pro Gly Phe Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys Arg Ala
115 120 125

Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg Asp Leu
130 135 140

Tyr Gln Leu Leu Ser Glu Arg Ile Ala Ile Leu His Pro Glu Gly Tyr
145 150 155 160

Leu Ile Thr Pro Ala Trp Leu Tyr Glu Lys Tyr Gly Leu Arg Pro Glu
165 170 175

Gln Trp Val Asp Tyr Arg Ala Leu Ala Gly Asp Pro Ser Asp Asn Ile
180 185 190

Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Gln Arg Leu Ile Arg
195 200 205

Glu Trp Gly Ser Leu Glu Asn Leu Phe Gln His Leu Asp Gln Val Lys
210 215 220

Pro Ser Leu Arg Glu Lys Leu Gln Ala Gly Met Glu Ala Leu Ala Leu
225 230 235 240

Ser Arg Lys Leu Ser Gln Val His Thr Asp Leu Pro Leu Glu Val Asp
245 250 255

Phe Gly Arg Arg Arg Thr Pro Asn Leu Glu Gly Leu Arg Ala Phe Leu
260 265 270

Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu Glu
275 280 285

Gly Pro Lys Ala Ala Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly Ala
290 295 300

Phe Leu Gly Phe Ser Phe Ser Arg Pro Glu Pro Met Trp Ala Glu Leu
305 310 315 320

Leu Ala Leu Ala Gly Ala Trp Glu Gly Arg Leu His Arg Ala Gln Asp
325 330 335

Pro Leu Arg Gly Leu Arg Asp Leu Lys Gly Val Arg Gly Ile Leu Ala
340 345 350

Lys Asp Leu Ala Val Leu Ala Leu Arg Glu Gly Leu Asp Leu Phe Pro
355 360 365

Glu Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr
370 375 380

Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Asp
385 390 395 400

Ala Gly Glu Arg Ala Leu Leu Ala Glu Arg Leu Phe Gln Thr Leu Lys
405 410 415

Glu Arg Leu Lys Gly Glu Glu Arg Leu Leu Trp Leu Tyr Glu Glu Val
420 425 430

Glu Lys Pro Leu Ser Arg Val Leu Ala Arg Met Glu Ala Thr Gly Val
435 440 445

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Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu Val Glu Ala
    450                 455                 460

Glu Val Arg Gln Leu Glu Glu Glu Val Phe Arg Leu Ala Gly His Pro
465                 470                 475                 480

Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu
                485                 490                 495

Leu Gly Leu Pro Ala Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser
            500                 505                 510

Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val
        515                 520                 525

Asp Arg Ile Leu Gln Tyr Arg Glu Leu Thr Lys Leu Lys Asn Thr Tyr
    530                 535                 540

Ile Asp Pro Leu Pro Ala Leu Val His Pro Lys Thr Gly Arg Leu His
545                 550                 555                 560

Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser
                565                 570                 575

Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg
            580                 585                 590

Ile Arg Arg Ala Phe Val Ala Glu Glu Gly Trp Val Leu Val Val Leu
        595                 600                 605

Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly Asp
    610                 615                 620

Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr Gln
625                 630                 635                 640

Thr Ala Ser Trp Met Phe Gly Val Ser Pro Glu Gly Val Asp Pro Leu
                645                 650                 655

Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly Met
            660                 665                 670

Ser Ala His Arg Leu Ser Gly Glu Leu Ser Ile Pro Tyr Glu Glu Ala
        675                 680                 685

Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Tyr Pro Lys Val Arg Ala
    690                 695                 700

Trp Ile Glu Gly Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val Glu
705                 710                 715                 720

Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn Ala Arg Val
                725                 730                 735

Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro Val
            740                 745                 750

Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Arg Leu Phe
        755                 760                 765

Pro Arg Leu Gln Glu Leu Gly Ala Arg Met Leu Leu Gln Val His Asp
    770                 775                 780

Glu Leu Val Leu Glu Ala Pro Lys Asp Arg Ala Glu Arg Val Ala Ala
785                 790                 795                 800
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Leu Ala Lys Glu Val Met Glu Gly Val Trp Pro Leu Gln Val Pro Leu
805 810 815

Glu Val Glu Val Gly Leu Gly Glu Asp Trp Leu Ser Ala Lys Glu
820 825 830

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 834 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
1 5 10 15

Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
20 25 30

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
35 40 45

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
50 55 60

Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
65 70 75 80

Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
85 90 95

Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
100 105 110

Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
115 120 125

Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
130 135 140

Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu
145 150 155 160

Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Arg
165 170 175

Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
180 185 190

Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
195 200 205

Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg
210 215 220

Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp
225 230 235 240

Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu
245 250 255

Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg
260 265 270

Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly
275 280 285

Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro
290 295 300

Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp
305 310 315 320

Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg
325 330 335

Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly
340 345 350

Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp
355 360 365

Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro
370 375 380

Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp
385 390 395 400

Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg
405 410 415

Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr
420 425 430

His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala
435 440 445

Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu
450 455 460

Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala
465 470 475 480

Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu
485 490 495

Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly
500 505 510

Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His
515 520 525

Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys
530 535 540

Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly
545 550 555 560

Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu
565 570 575

Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu
580 585 590

Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu
595 600 605

Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu
610 615 620

Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile
625 630 635 640

His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val
645 650 655

Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu
660 665 670

Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr
675 680 685

Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys
690 695 700

Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly
705 710 715 720

Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn
725 730 735

Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn
740 745 750

Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val
755 760 765

Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
770 775 780

Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
785 790 795 800

Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
805 810 815

Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala
820 825 830

Lys Gly

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2502 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGNNGGCGA TGCTTCCCCT CTTTGAGCCC AAAGGCCGGG TCCTCCTGGT GGACGGCCAC 60

CACCTGGCCT ACCGCACCTT CTTCGCCCTG AAGGGCCTCA CCACCAGCCG GGGCGAACCG 120

GTGCAGGCGG TCTACGGCTT CGCCAAGAGC CTCCTCAAGG CCCTGAAGGA GGACGGGGAC 180

NNGGCGGTGN TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGAG 240

GCCTACAAGG CGGGCCGGGC CCCCACCCCG GAGGACTTTC CCCGGCAGCT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGCTTGCG CGCCTCGAGG TCCCCGGCTA CGAGGCGGAC 360

GACGTNCTGG CCACCCTGGC CAAGAAGGCG GAAAAGGAGG GGTACGAGGT GCGCATCCTC 420

ACCGCCGACC GCGACCTCTA CCAGCTCCTT TCCGACCGCA TCGCCGTCCT CCACCCCGAG 480

GGGTACCTCA TCACCCCGGC GTGGCTTTGG GAGAAGTACG GCCTGAGGCC GGAGCAGTGG 540

GTGGACTACC GGGCCCTGGC GGGGGACCCC TCCGACAACC TCCCCGGGGT CAAGGGCATC 600

GGGGAGAAGA CCGCCCNGAA GCTCCTCNAG GAGTGGGGGA GCCTGGAAAA CCTCCTCAAG 660

AACCTGGACC GGGTGAAGCC CGCCNTCCGG GAGAAGATCC AGGCCCACAT GGANGACCTG 720

ANGCTCTCCT GGGAGCTNTC CCAGGTGCGC ACCGACCTGC CCCTGGAGGT GGACTTCGCC 780

AAGNGGCGGG AGCCCGACCG GGAGGGGCTT AGGGCCTTTC TGGAGAGGCT GGAGTTTGGC 840

AGCCTCCTCC ACGAGTTCGG CCTCCTGGAG GGCCCCAAGG CCCTGGAGGA GGCCCCCTGG 900

CCCCCGCCGG AAGGGGCCTT CGTGGGCTTT GTCCTTTCCC GCCCCGAGCC CATGTGGGCC 960

GAGCTTCTGG CCCTGGCCGC CGCCAGGGAG GGCCGGGTCC ACCGGGCACC AGACCCCTTT 1020

ANGGGCCTNA GGGACCTNAA GGAGGTGCGG GGNCTCCTCG CCAAGGACCT GGCCGTTTTG 1080

GCCCTGAGGG AGGGCCTNGA CCTCNTGCCC GGGGACGACC CCATGCTCCT CGCCTACCTC 1140

CTGGACCCCT CCAACACCAC CCCCGAGGGG GTGGCCCGGC GCTACGGGGG GGAGTGGACG 1200

GAGGANGCGG GGGAGCGGGC CCTCCTNTCC GAGAGGCTCT TCCNGAACCT NNNGCAGCGC 1260

CTTGAGGGGG AGGAGAGGCT CCTTTGGCTT TACCAGGAGG TGGAGAAGCC CCTTTCCCGG 1320

GTCCTGGCCC ACATGGAGGC CACGGGGGTN CGGCTGGACG TGGCCTACCT CCAGGCCCTN 1380

TCCCTGGAGG TGGCGGAGGA GATCCGCCGC CTCGAGGAGG AGGTCTTCCG CCTGGCCGGC 1440

CACCCCTTCA ACCTCAACTC CCGGGACCAG CTGGAAAGGG TGCTCTTTGA CGAGCTNGGG 1500

CTTCCCGCCA TCGGCAAGAC GGAGAAGACN GGCAAGCGCT CCACCAGCGC CGCCGTGCTG 1560

GAGGCCCTNC GNGAGGCCCA CCCCATCGTG GAGAAGATCC TGCAGTACCG GGAGCTCACC 1620

AAGCTCAAGA ACACCTACAT NGACCCCCTG CCNGNCCTCG TCCACCCCAG GACGGGCCGC 1680

CTCCACACCC GCTTCAACCA GACGGCCACG GCCACGGGCA GGCTTAGTAG CTCCGACCCC 1740

AACCTGCAGA ACATCCCCGT CCGCACCCCN CTGGGCCAGA GGATCCGCCG GGCCTTCGTG 1800

GCCGAGGAGG GNTGGGTGTT GGTGGCCCTG GACTATAGCC AGATAGAGCT CCGGGTCCTG 1860

GCCCACCTCT CCGGGGACGA GAACCTGATC CGGGTCTTCC AGGAGGGGAG GGACATCCAC 1920

ACCCAGACCG CCAGCTGGAT GTTCGGCGTC CCCCCGGAGG CCGTGGACCC CCTGATGCGC 1980

CGGGCGGCCA AGACCATCAA CTTCGGGGTC CTCTACGGCA TGTCCGCCCA CCGCCTCTCC 2040

CAGGAGCTTG CCATCCCCTA CGAGGAGGCG GTGGCCTTCA TTGAGCGCTA CTTCCAGAGC 2100

TTCCCCAAGG TGCGGGCCTG GATTGAGAAG ACCCTGGAGG AGGGCAGGAG GCGGGGGTAC 2160

GTGGAGACCC TCTTCGGCCG CCGGCGCTAC GTGCCCGACC TCAACGCCCG GGTGAAGAGC 2220

GTGCGGGAGG CGGCGGAGCG CATGGCCTTC AACATGCCCG TCCAGGGCAC CGCCGCCGAC 2280

CTCATGAAGC TGGCCATGGT GAAGCTCTTC CCCCGGCTNC AGGAAATGGG GGCCAGGATG 2340

CTCCTNCAGG TCCACGACGA GCTGGTCCTC GAGGCCCCCA AAGAGCGGGC GGAGGNGGTG 2400

GCCGCTTTGG CCAAGGAGGT CATGGAGGGG GTCTATCCCC TGGCCGTGCC CCTGGAGGTG 2460

GAGGTGGGGA TGGGGGAGGA CTGGCTCTCC GCCAAGGAGT AG 2502

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 833 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Xaa Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
1 5 10 15

Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
20 25 30

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
35 40 45

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Xaa Val
50 55 60

Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu Ala
65 70 75 80

Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu
85 90 95

Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Xaa Arg Leu Glu
100 105 110

Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys Lys
115 120 125

Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg Asp
130 135 140

Leu Tyr Gln Leu Leu Ser Asp Arg Ile Ala Val Leu His Pro Glu Gly
145 150 155 160

Tyr Leu Ile Thr Pro Ala Trp Leu Trp Glu Lys Tyr Gly Leu Arg Pro
165 170 175

Glu Gln Trp Val Asp Tyr Arg Ala Leu Xaa Gly Asp Pro Ser Asp Asn
180 185 190

Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Xaa Lys Leu Leu
195 200 205

Xaa Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg Val
210 215 220

Lys Pro Xaa Xaa Arg Glu Lys Ile Xaa Ala His Met Glu Asp Leu Xaa
225 230 235 240

Leu Ser Xaa Xaa Leu Ser Xaa Val Arg Thr Asp Leu Pro Leu Glu Val
245 250 255

Asp Phe Ala Xaa Arg Arg Glu Pro Asp Arg Glu Gly Leu Arg Ala Phe
260 265 270

Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu
275 280 285

Glu Xaa Pro Lys Ala Leu Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly
290 295 300

Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp Ala Glu
305 310 315 320

Leu Leu Ala Leu Ala Ala Ala Arg Xaa Gly Arg Val His Arg Ala Xaa
325 330 335

Asp Pro Leu Xaa Gly Leu Arg Asp Leu Lys Glu Val Arg Gly Leu Leu
340 345 350

Ala Lys Asp Leu Ala Val Leu Ala Leu Arg Glu Gly Leu Asp Leu Xaa
355 360 365

Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn
370 375 380

Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu
385 390 395 400

Asp Ala Gly Glu Arg Ala Leu Leu Ser Glu Arg Leu Phe Xaa Asn Leu
405 410 415

Xaa Xaa Arg Leu Glu Gly Glu Glu Arg Leu Leu Trp Leu Tyr Xaa Glu
420 425 430

Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala Thr Gly
435 440 445

Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu Val Ala
450 455 460

Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala Gly His
465 470 475 480

Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp
485 490 495

Glu Leu Gly Leu Pro Ala Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg
500 505 510

Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile
515 520 525

Val Glu Lys Ile Leu Gln Tyr Arg Glu Leu Thr Lys Leu Lys Asn Thr
530 535 540

Tyr Ile Asp Pro Leu Pro Xaa Leu Val His Pro Arg Thr Gly Arg Leu
545 550 555 560

His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser
565 570 575

Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln
580 585 590

Arg Ile Arg Arg Ala Phe Val Ala Glu Glu Gly Trp Xaa Leu Val Ala
595 600 605

Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly
610 615 620

Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr
625 630 635 640

Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val Asp Pro
645 650 655

Leu Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly
660 665 670

Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
675 680 685

Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg
690 695 700

Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val
705 710 715 720

Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn Ala Arg
725 730 735

Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro
740 745 750

Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu
755 760 765

Phe Pro Arg Leu Xaa Glu Met Gly Ala Arg Met Leu Leu Gln Val His
770 775 780

Asp Glu Leu Val Leu Glu Ala Pro Lys Xaa Arg Ala Glu Xaa Val Ala
785 790 795 800

Ala Leu Ala Lys Glu Val Met Glu Gly Val Tyr Pro Leu Ala Val Pro
805 810 815

Leu Glu Val Glu Val Gly Xaa Gly Glu Asp Trp Leu Ser Ala Lys Glu
820 825 830

Xaa

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1647 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAATTCGG GGATGCTGCC CCTCTTTGAG CCCAAGGGCC GGGTCCTCCT GGTGGACGGC 60

CACCACCTGG CCTACCGCAC CTTCCACGCC CTGAAGGGCC TCACCACCAG CCGGGGGGAG 120

CCGGTGCAGG CGGTCTACGG CTTCGCCAAG AGCCTCCTCA AGGCCCTCAA GGAGGACGGG 180

GACGCGGTGA TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGGG 240

GGGTACAAGG CGGGCCGGGC CCCCACGCCG GAGGACTTTC CCCGGCAACT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGCTGGCG CGCCTCGAGG TCCCGGGCTA CGAGGCGGAC 360

GACGTCCTGG CCAGCCTGGC CAAGAAGGCG GAAAAGGAGG GCTACGAGGT CCGCATCCTC 420

ACCGCCGACA AAGACCTTTA CCAGCTCCTT TCCGACCGCA TCCACGTCCT CCACCCCGAG 480

GGGTACCTCA TCACCCCGGC CTGGCTTTGG GAAAAGTACG GCCTGAGGCC CGACCAGTGG 540

GCCGACTACC GGGCCCTGAC CGGGGACGAG TCCGACAACC TTCCCGGGGT CAAGGGCATC 600

GGGGAGAAGA CGGCGAGGAA GCTTCTGGAG GAGTGGGGGA GCCTGGAAGC CCTCCTCAAG 660

AACCTGGACC GGCTGAAGCC CGCCATCCGG GAGAAGATCC TGGCCCACAT GGACGATCTG 720

AAGCTCTCCT GGGACCTGGC CAAGGTGCGC ACCGACCTGC CCCTGGAGGT GGACTTCGCC 780

AAAAGGCGGG AGCCCGACCG GGAGAGGCTT AGGGCCTTTC TGGAGAGGCT TGAGTTTGGC 840

AGCCTCCTCC ACGAGTTCGG CCTTCTGGAA AGCCCCAAGG CCCTGGAGGA GGCCCCCTGG 900

CCCCCGCCGG AAGGGGCCTT CGTGGGCTTT GTGCTTTCCC GCAAGGAGCC CATGTGGGCC 960

GATCTTCTGG CCCTGGCCGC CGCCAGGGGG GGCCGGGTCC ACCGGGCCCC CGAGCCTTAT 1020

AAAGCCCTCA GGGACCTGAA GGAGGCGCGG GGGCTTCTCG CCAAAGACCT GAGCGTTCTG 1080

GCCCTGAGGG AAGGCCTTGG CCTCCCGCCC GGCGACGACC CCATGCTCCT CGCCTACCTC 1140

CTGGACCCTT CCAACACCAC CCCCGAGGGG GTGGCCCGGC GCTACGGCGG GGAGTGGACG 1200

GAGGAGGCGG GGGAGCGGGC CGCCCTTTCC GAGAGGCTCT TCGCCAACCT GTGGGGGAGG 1260

CTTGAGGGGG AGGAGAGGCT CCTTTGGCTT TACCGGGAGG TGGAGAGGCC CCTTTCCGCT 1320

GTCCTGGCCC ACATGGAGGC CACGGGGGTG CGCCTGGACG TGGCCTATCT CAGGGCCTTG 1380

TCCCTGGAGG TGGCCGGGGA GATCGCCCGC CTCGAGGCCG AGGTCTTCCG CCTGGCCGGC 1440

CACCCCTTCA ACCTCAACTC CCGGGACCAG CTGGAAAGGG TCCTCTTTGA CGAGCTAGGG 1500

CTTCCCGCCA TCGGCAAGAC GGAGAAGACC GGCAAGCGCT CCACCAGCGC CGCCGTCCTG 1560

GAGGCCCTCC GCGAGGCCCA CCCCATCGTG GAGAAGATCC TGCAGGCATG CAAGCTTGGC 1620

ACTGGCCGTC GTTTTACAAC GTCGTGA 1647

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2088 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAATTCGG GGATGCTGCC CCTCTTTGAG CCCAAGGGCC GGGTCCTCCT GGTGGACGGC 60

CACCACCTGG CCTACCGCAC CTTCCACGCC CTGAAGGGCC TCACCACCAG CCGGGGGGAG 120

CCGGTGCAGG CGGTCTACGG CTTCGCCAAG AGCCTCCTCA AGGCCCTCAA GGAGGACGGG 180

GACGCGGTGA TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGGG 240

GGGTACAAGG CGGGCCGGGC CCCCACGCCG GAGGACTTTC CCCGGCAACT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGCTGGCG CGCCTCGAGG TCCCGGGCTA CGAGGCGGAC 360

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GACGTCCTGG CCAGCCTGGC CAAGAAGGCG GAAAAGGAGG GCTACGAGGT CCGCATCCTC     420
ACCGCCGACA AAGACCTTTA CCAGCTCCTT TCCGACCGCA TCCACGTCCT CCACCCCGAG     480
GGGTACCTCA TCACCCCGGC CTGGCTTTGG GAAAAGTACG GCCTGAGGCC CGACCAGTGG     540
GCCGACTACC GGGCCCTGAC CGGGGACGAG TCCGACAACC TTCCCGGGGT CAAGGGCATC     600
GGGGAGAAGA CGGCGAGGAA GCTTCTGGAG GAGTGGGGGA GCCTGGAAGC CCTCCTCAAG     660
AACCTGGACC GGCTGAAGCC CGCCATCCGG GAGAAGATCC TGGCCCACAT GGACGATCTG     720
AAGCTCTCCT GGGACCTGGC CAAGGTGCGC ACCGACCTGC CCCTGGAGGT GGACTTCGCC     780
AAAAGGCGGG AGCCCGACCG GGAGAGGCTT AGGGCCTTTC TGGAGAGGCT TGAGTTTGGC     840
AGCCTCCTCC ACGAGTTCGG CCTTCTGGAA AGCCCCAAGG CCCTGGAGGA GGCCCCCTGG     900
CCCCCGCCGG AAGGGGCCTT CGTGGGCTTT GTGCTTTCCC GCAAGGAGCC CATGTGGGCC     960
GATCTTCTGG CCCTGGCCGC CGCCAGGGGG GGCCGGGTCC ACCGGGCCCC CGAGCCTTAT    1020
AAAGCCCTCA GGGACCTGAA GGAGGCGCGG GGGCTTCTCG CCAAAGACCT GAGCGTTCTG    1080
GCCCTGAGGG AAGGCCTTGG CCTCCCGCCC GGCGACGACC CCATGCTCCT CGCCTACCTC    1140
CTGGACCCTT CCAACACCAC CCCCGAGGGG GTGGCCCGGC GCTACGGCGG GGAGTGGACG    1200
GAGGAGGCGG GGGAGCGGGC CGCCCTTTCC GAGAGGCTCT TCGCCAACCT GTGGGGGAGG    1260
CTTGAGGGGG AGGAGAGGCT CCTTTGGCTT TACCGGGAGG TGGAGAGGCC CCTTTCCGCT    1320
GTCCTGGCCC ACATGGAGGC CACGGGGGTG CGCCTGGACG TGGCCTATCT CAGGGCCTTG    1380
TCCCTGGAGG TGGCCGGGGA GATCGCCCGC CTCGAGGCCG AGGTCTTCCG CCTGGCCGGC    1440
CACCCCTTCA ACCTCAACTC CCGGGACCAG CTGGAAAGGG TCCTCTTTGA CGAGCTAGGG    1500
CTTCCCGCCA TCGGCAAGAC GGAGAAGACC GGCAAGCGCT CCACCAGCGC CGCCGTCCTG    1560
GAGGCCCTCC GCGAGGCCCA CCCCATCGTG GAGAAGATCC TGCAGTACCG GGAGCTCACC    1620
AAGCTGAAGA GCACCTACAT TGACCCCTTG CCGGACCTCA TCCACCCCAG GACGGGCCGC    1680
CTCCACACCC GCTTCAACCA GACGGCCACG GCCACGGGCA GGCTAAGTAG CTCCGATCCC    1740
AACCTCCAGA ACATCCCCGT CCGCACCCCG CTTGGGCAGA GGATCCGCCG GGCCTTCATC    1800
GCCGAGGAGG GGTGGCTATT GGTGGCCCTG GACTATAGCC AGATAGAGCT CAGGGTGCTG    1860
GCCCACCTCT CCGGCGACGA GAACCTGATC CGGGTCTTCC AGGAGGGGCG GGACATCCAC    1920
ACGGAGACCG CCAGCTGGAT GTTCGGCGTC CCCCGGGAGG CCGTGGACCC CCTGATGCGC    1980
CGGGCGGCCA AGACCATCAA CTTCGGGGTC CTCTACGGCA TGTCGGCCCA CCGCCTCTCC    2040
CAGGAGCTAG CTAGCCATCC CTTACGAGGA GGCCCAGGCC TTCATTGA                  2088
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 962 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGAATTCGG GGATGCTGCC CCTCTTTGAG CCCAAGGGCC GGGTCCTCCT GGTGGACGGC 60

CACCACCTGG CCTACCGCAC CTTCCACGCC CTGAAGGGCC TCACCACCAG CCGGGGGGAG 120

CCGGTGCAGG CGGTCTACGG CTTCGCCAAG AGCCTCCTCA AGGCCCTCAA GGAGGACGGG 180

GACGCGGTGA TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGGG 240

GGGTACAAGG CGGGCCGGGC CCCCACGCCG GAGGACTTTC CCCGGCAACT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGCTGGCG CGCCTCGAGG TCCCGGGCTA CGAGGCGGAC 360

GACGTCCTGG CCAGCCTGGC CAAGAAGGCG GAAAAGGAGG GCTACGAGGT CCGCATCCTC 420

ACCGCCGACA AAGACCTTTA CCAGCTTCTT TCCGACCGCA TCCACGTCCT CCACCCCGAG 480

GGGTACCTCA TCACCCCGGC CTGGCTTTGG GAAAAGTACG GCCTGAGGCC CGACCAGTGG 540

GCCGACTACC GGGCCCTGAC CGGGGACGAG TCCGACAACC TTCCCGGGGT CAAGGGCATC 600

GGGGAGAAGA CGGCGAGGAA GCTTCTGGAG GAGTGGGGGA GCCTGGAAGC CCTCCTCAAG 660

AACCTGGACC GGCTGAAGCC CGCCATCCGG GAGAAGATCC TGGCCCACAT GGACGATCTG 720

AAGCTCTCCT GGGACCTGGC CAAGGTGCGC ACCGACCTGC CCCTGGAGGT GGACTTCGCC 780

AAAAGGCGGG AGCCCGACCG GGAGAGGCTT AGGGCCTTTC TGGAGAGGCT TGAGTTTGGC 840

AGCCTCCTCC ACGAGTTCGG CCTTCTGGAA AGCCCCAAGT CATGGAGGGG GTGTATCCCC 900

TGGCCGTGCC CCTGGAGGTG GAGGTGGGGA TAGGGGAGGA CTGGCTCTCC GCCAAGGAGT 960

GA 962

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGGAATTCG GGGATGCTGC CCCTCTTTGA GCCCAAGGGC CGGGTCCTCC TGGTGGACGG 60

CCACCACCTG GCCTACCGCA CCTTCCACGC CCTGAAGGGC CTCACCACCA GCCGGGGGGA 120

GCCGGTGCAG GCGGTCTACG GCTTCGCCAA GAGCCTCCTC AAGGCCCTCA AGGAGGACGG 180

GGACGCGGTG ATCGTGGTCT TTGACGCCAA GGCCCCCTCC TTCCGCCACG AGGCCTACGG 240

GGGGTACAAG GCGGGCCGGG CCCCCACGCC GGAGGACTTT CCCCGGCAAC TCGCCCTCAT 300

CAAGGAGCTG GTGGACCTCC TGGGGCTGGC GCGCCTCGAG GTCCCGGGCT ACGAGGCGGA 360

CGACGTCCTG GCCAGCCTGG CCAAGAAGGC GGAAAAGGAG GGCTACGAGG TCCGCATCCT 420

CACCGCCGAC AAAGACCTTT ACCAGCTCCT TTCCGACCGC ATCCACGTCC TCCACCCCGA 480

GGGGTACCTC ATCACCCCGG CCTGGCTTTG GGAAAAGTAC GGCCTGAGGC CCGACCAGTG 540

GGCCGACTAC CGGGCCCTGA CCGGGGACGA GTCCGACAAC CTTCCCGGGG TCAAGGGCAT 600

CGGGGAGAAG ACGGCGAGGA AGCTTCTGGA GGAGTGGGGG AGCCTGGAAG CCCTCCTCAA 660

GAACCTGGAC CGGCTGAAGC CCGCCATCCG GGAGAAGATC CTGGCCCACA TGGACGATCT 720

GAAGCTCTCC TGGGACCTGG CCAAGGTGCG CACCGACCTG CCCCTGGAGG TGGACTTCGC 780

CAAAAGGCGG GAGCCCGACC GGGAGAGGCT TAGGGCCTTT CTGGAGAGGC TTGAGTTTGG 840

CAGCCTCCTC CACGAGTTCG GCCTTCTGGA AAGCCCCAAG ATCCGCCGGG CCTTCATCGC 900

CGAGGAGGGG TGGCTATTGG TGGCCCTGGA CTATAGCCAG ATAGAGCTCA GGGTGCTGGC 960

CCACCTCTCC GGCGACGAGA ACCTGATCCG GGTCTTCCAG GAGGGGCGGG ACATCCACAC 1020

GGAGACCGCC AGCTGGATGT TCGGCGTCCC CCGGGAGGCC GTGGACCCCC TGATGCGCCG 1080

GGCGGCCAAG ACCATCAACT TCGGGGTCCT CTACGGCATG TCGGCCCACC GCCTCTCCCA 1140

GGAGCTAGCC ATCCCTTACG AGGAGGCCCA GGCCTTCATT GAGCGCTACT TTCAGAGCTT 1200

CCCCAAGGTG CGGGCCTGGA TTGAGAAGAC CCTGGAGGAG GGCAGGAGGC GGGGGTACGT 1260

GGAGACCCTC TTCGGCCGCC GCCGCTACGT GCCAGACCTA GAGGCCCGGG TGAAGAGCGT 1320

GCGGGAGGCG GCCGAGCGCA TGGCCTTCAA CATGCCCGTC CGGGGCACCG CCGCCGACCT 1380

CATGAAGCTG GCTATGGTGA AGCTCTTCCC CAGGCTGGAG GAAATGGGGG CCAGGATGCT 1440

CCTTCAGGTC CACGACGAGC TGGTCCTCGA GGCCCCAAAA GAGAGGGCGG AGGCCGTGGC 1500

CCGGCTGGCC AAGGAGGTCA TGGAGGGGGT GTATCCCCTG GCCGTGCCCC TGGAGGTGGA 1560

GGTGGGGATA GGGGAGGACT GGCTCTCCGC CAAGGAGTGA 1600

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACGAATTCG GGGATGCTGC CCCTCTTTGA GCCCAA 36

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGAGATCTA TCACTCCTTG GCGGAGAGCC AGTC 34

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TAATACGACT CACTATAGGG AGACCGGAAT TCGAGCTCGC CCGGGCGAGC TCGAATTCCG 60

TGTATTCTAT AGTGTCACCT AAATCGAATT C 91

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAATTCGATT TAGGTGACAC TATAGAA 27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTAATCATGG TCATAGCTGG TAGCTTGCTA C 31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATCCTCTA GAGTCGACCT GCAGGCATGC CTACCTTGGT AG 42

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGATCCTCTA GAGTCGACCT GCAGGCATGC 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2502 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATGAATTCGG GGATGCTGCC CCTCTTTGAG CCCAAGGGCC GGGTCCTCCT GGTGGACGGC 60

CACCACCTGG CCTACCGCAC CTTCCACGCC CTGAAGGGCC TCACCACCAG CCGGGGGGAG 120

CCGGTGCAGG CGGTCTACGG CTTCGCCAAG AGCCTCCTCA AGGCCCTCAA GGAGGACGGG 180

GACGCGGTGA TCGTGGTCTT TGACGCCAAG GCCCCCTCCT TCCGCCACGA GGCCTACGGG 240

GGGTACAAGG CGGGCCGGGC CCCCACGCCG GAGGACTTTC CCCGGCAACT CGCCCTCATC 300

AAGGAGCTGG TGGACCTCCT GGGGCTGGCG CGCCTCGAGG TCCCGGGCTA CGAGGCGGAC 360

GACGTCCTGG CCAGCCTGGC CAAGAAGGCG GAAAAGGAGG GCTACGAGGT CCGCATCCTC 420

ACCGCCGACA AAGACCTTTA CCAGCTCCTT TCCGACCGCA TCCACGTCCT CCACCCCGAG 480

GGGTACCTCA TCACCCCGGC CTGGCTTTGG GAAAAGTACG GCCTGAGGCC CGACCAGTGG 540

GCCGACTACC GGGCCCTGAC CGGGGACGAG TCCGACAACC TTCCCGGGGT CAAGGGCATC 600

GGGGAGAAGA CGGCGAGGAA GCTTCTGGAG GAGTGGGGGA GCCTGGAAGC CCTCCTCAAG 660

AACCTGGACC GGCTGAAGCC CGCCATCCGG GAGAAGATCC TGGCCCACAT GGACGATCTG 720

AAGCTCTCCT GGGACCTGGC CAAGGTGCGC ACCGACCTGC CCCTGGAGGT GGACTTCGCC 780

AAAAGGCGGG AGCCCGACCG GGAGAGGCTT AGGGCCTTTC TGGAGAGGCT TGAGTTTGGC 840

AGCCTCCTCC ACGAGTTCGG CCTTCTGGAA AGCCCCAAGG CCCTGGAGGA GGCCCCCTGG 900

CCCCCGCCGG AAGGGGCCTT CGTGGGCTTT GTGCTTTCCC GCAAGGAGCC CATGTGGGCC 960

GATCTTCTGG CCCTGGCCGC CGCCAGGGGG GGCCGGGTCC ACCGGGCCCC CGAGCCTTAT 1020

AAAGCCCTCA GGGACCTGAA GGAGGCGCGG GGGCTTCTCG CCAAAGACCT GAGCGTTCTG 1080

GCCCTGAGGG AAGGCCTTGG CCTCCCGCCC GGCGACGACC CCATGCTCCT CGCCTACCTC 1140

CTGGACCCTT CCAACACCAC CCCCGAGGGG GTGGCCCGGC GCTACGGCGG GGAGTGGACG 1200

GAGGAGGCGG GGGAGCGGGC CGCCCTTTCC GAGAGGCTCT TCGCCAACCT GTGGGGGAGG 1260

CTTGAGGGGG AGGAGAGGCT CCTTTGGCTT TACCGGGAGG TGGAGAGGCC CCTTTCCGCT 1320

GTCCTGGCCC ACATGGAGGC CACGGGGGTG CGCCTGGACG TGGCCTATCT CAGGGCCTTG 1380

TCCCTGGAGG TGGCCGGGGA GATCGCCCGC CTCGAGGCCG AGGTCTTCCG CCTGGCCGGC 1440

CACCCCTTCA ACCTCAACTC CCGGGACCAG CTGGAAAGGG TCCTCTTTGA CGAGCTAGGG 1500

CTTCCCGCCA TCGGCAAGAC GGAGAAGACC GGCAAGCGCT CCACCAGCGC CGCCGTCCTG 1560

GAGGCCCTCC GCGAGGCCCA CCCCATCGTG GAGAAGATCC TGCAGTACCG GGAGCTCACC 1620

AAGCTGAAGA GCACCTACAT TGACCCCTTG CCGGACCTCA TCCACCCCAG GACGGGCCGC 1680

CTCCACACCC GCTTCAACCA GACGGCCACG GCCACGGGCA GGCTAAGTAG CTCCGATCCC 1740

AACCTCCAGA ACATCCCCGT CCGCACCCCG CTTGGGCAGA GGATCCGCCG GGCCTTCATC 1800

GCCGAGGAGG GGTGGCTATT GGTGGCCCTG GACTATAGCC AGATAGAGCT CAGGGTGCTG 1860

GCCCACCTCT CCGGCGACGA GAACCTGATC CGGGTCTTCC AGGAGGGGCG GGACATCCAC 1920

ACGGAGACCG CCAGCTGGAT GTTCGGCGTC CCCCGGGAGG CCGTGGACCC CCTGATGCGC 1980

CGGGCGGCCA AGACCATCAA CTTCGGGGTC CTCTACGGCA TGTCGGCCCA CCGCCTCTCC 2040

CAGGAGCTAG CCATCCCTTA CGAGGAGGCC CAGGCCTTCA TTGAGCGCTA CTTTCAGAGC 2100

TTCCCCAAGG TGCGGGCCTG GATTGAGAAG ACCCTGGAGG AGGGCAGGAG GCGGGGGTAC 2160

GTGGAGACCC TCTTCGGCCG CCGCCGCTAC GTGCCAGACC TAGAGGCCCG GGTGAAGAGC 2220

GTGCGGGAGG CGGCCGAGCG CATGGCCTTC AACATGCCCG TCCGGGGCAC CGCCGCCGAC 2280

CTCATGAAGC TGGCTATGGT GAAGCTCTTC CCCAGGCTGG AGGAAATGGG GGCCAGGATG 2340

CTCCTTCAGG TCCACGACGA GCTGGTCCTC GAGGCCCCAA AAGAGAGGGC GGAGGCCGTG 2400

GCCCGGCTGG CCAAGGAGGT CATGGAGGGG GTGTATCCCC TGGCCGTGCC CCTGGAGGTG 2460

GAGGTGGGGA TAGGGGAGGA CTGGCTCTCC GCCAAGGAGT GA 2502

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATTTAGGTG ACACTATAG 19

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGGACGAACA AGCGAGACAG CGACACAGGT ACCACATGGT ACAAGAGGCA AGAGAGACGA 60

CACAGCAGAA AC 72

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTTCTGCTG TGTCGTCTCT CTTGCCTCTT GTACCATGTG GTACCTGTGT CGCTGTCTCG 60

CTTGTTCGTC 70

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GACGAACAAG CGAGACAGCG 20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTTCTGCTG TGTCGTCTCT CTTG 24

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCTCTTGTAC CATGTGGTAC CTGTGTCGCT GTCTCGCTTG TTCGTC 46

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACACAGGTAC CACATGGTAC AAGAGGCAAG AGAGACGACA CAGCAGAAAC 50

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Ile Asn Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 969 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGGCTAGCA TGACTGGTGG ACAGCAAATG GGTCGGATCA ATTCGGGGAT GCTGCCCCTC 60

TTTGAGCCCA AGGGCCGGGT CCTCCTGGTG GACGGCCACC ACCTGGCCTA CCGCACCTTC 120

CACGCCCTGA AGGGCCTCAC CACCAGCCGG GGGGAGCCGG TGCAGGCGGT CTACGGCTTC 180

GCCAAGAGCC TCCTCAAGGC CCTCAAGGAG GACGGGGACG CGGTGATCGT GGTCTTTGAC 240

GCCAAGGCCC CCTCCTTCCG CCACGAGGCC TACGGGGGGT ACAAGGCGGG CCGGGCCCCC 300

ACGCCGGAGG ACTTTCCCCG GCAACTCGCC CTCATCAAGG AGCTGGTGGA CCTCCTGGGG 360

CTGGCGCGCC TCGAGGTCCC GGGCTACGAG GCGGACGACG TCCTGGCCAG CCTGGCCAAG 420

AAGGCGGAAA AGGAGGGCTA CGAGGTCCGC ATCCTCACCG CCGACAAAGA CCTTTACCAG 480

CTTCTTTCCG ACCGCATCCA CGTCCTCCAC CCCGAGGGGT ACCTCATCAC CCCGGCCTGG 540

CTTTGGGAAA AGTACGGCCT GAGGCCCGAC CAGTGGGCCG ACTACCGGGC CCTGACCGGG 600

GACGAGTCCG ACAACCTTCC CGGGGTCAAG GGCATCGGGG AGAAGACGGC GAGGAAGCTT 660

CTGGAGGAGT GGGGGAGCCT GGAAGCCCTC CTCAAGAACC TGGACCGGCT GAAGCCCGCC 720

ATCCGGGAGA AGATCCTGGC CCACATGGAC GATCTGAAGC TCTCCTGGGA CCTGGCCAAG 780

GTGCGCACCG ACCTGCCCCT GGAGGTGGAC TTCGCCAAAA GGCGGGAGCC CGACCGGGAG 840

AGGCTTAGGG CCTTTCTGGA GAGGCTTGAG TTTGGCAGCC TCCTCCACGA GTTCGGCCTT 900

GTGGAAAGCC CCAAGTCATG GAGGGGGTGT ATCCCCTGGC CGTGCCCCTG GAGGTGGAGG 960

TGGGGATAG 969

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 948 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ATGGCTAGCA TGACTGGTGG ACAGCAAATG GGTCGGATCA ATTCGGGGAT GCTGCCCCTC 60

TTTGAGCCCA AGGGCCGGGT CCTCCTGGTG GACGGCCACC ACCTGGCCTA CCGCACCTTC 120

CACGCCCTGA AGGGCCTCAC CACCAGCCGG GGGGAGCCGG TGCAGGCGGT CTACGGCTTC 180

GCCAAGAGCC TCCTCAAGGC CCTCAAGGAG GACGGGGACG CGGTGATCGT GGTCTTTGAC 240

GCCAAGGCCC CCTCCTTCCG CCACGAGGCC TACGGGGGGT ACAAGGCGGG CCGGGCCCCC 300

ACGCCGGAGG ACTTTCCCCG GCAACTCGCC CTCATCAAGG AGCTGGTGGA CCTCCTGGGG 360

CTGGCGCGCC TCGAGGTCCC GGGCTACGAG GCGGACGACG TCCTGGCCAG CCTGGCCAAG 420

AAGGCGGAAA AGGAGGGCTA CGAGGTCCGC ATCCTCACCG CCGACAAAGA CCTTTACCAG 480

CTTCTTTCCG ACCGCATCCA CGTCCTCCAC CCCGAGGGGT ACCTCATCAC CCCGGCCTGG 540

CTTTGGGAAA AGTACGGCCT GAGGCCCGAC CAGTGGGCCG ACTACCGGGC CCTGACCGGG 600

GACGAGTCCG ACAACCTTCC CGGGGTCAAG GGCATCGGGG AGAAGACGGC GAGGAAGCTT 660

CTGGAGGAGT GGGGGAGCCT GGAAGCCCTC CTCAAGAACC TGGACCGGCT GAAGCCCGCC 720

ATCCGGGAGA AGATCCTGGC CCACATGGAC GATCTGAAGC TCTCCTGGGA CCTGGCCAAG 780

GTGCGCACCG ACCTGCCCCT GGAGGTGGAC TTCGCCAAAA GGCGGGAGCC CGACCGGGAG 840

AGGCTTAGGG CCTTTCTGGA GAGGCTTGAG TTTGGCAGCC TCCTCCACGA GTTCGGCCTT 900

CTGGAAAGCC CCAAGGCCGC ACTCGAGCAC CACCACCACC ACCACTGA 948

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 206 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGCCAGGGTT TTCCCAGTCA CGACGTTGTA AAACGACGGC CAGTGAATTG TAATACGACT 60

CACTATAGGG CGAATTCGAG CTCGGTACCC GGGGATCCTC TAGAGTCGAC CTGCAGGCAT 120

GCAAGCTTGA GTATTCTATA GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG 180

TTTCCTGTGT GAAATTGTTA TCCGCT 206

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TTCTGGGTTC TCTGCTCTCT GGTCGCTGTC TCGCTTGTTC GTC 43

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCTGTCTCGC TTGTTCGTC 19

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GACGAACAAG CGAGACAGCG 20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TTCTGGGTTC TCTGCTCTCT GGTC 24

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GACGAACAAG CGAGACAGCG ACCAGAGAGC AGAGAACCCA GAA 43

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ACCAGAGAGC AGAGAACCCA GAA 23

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AACAGCTATG ACCATGATTA C 21

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGATCCTCTA GAGTCGACCT GCAGGCATGC 30

CLAIMS

We claim:

1. A DNA sequence encoding a thermostable DNA polymerase altered in sequence relative to the native sequence such that it exhibits altered DNA synthetic activity from that of the native DNA polymerase.

2. The DNA sequence of Claim 1 wherein the encoded DNA polymerase exhibits reduced synthetic activity from that of the native DNA polymerase.

3. The DNA sequence of Claim 1 wherein the alteration to said native sequence comprises a change in a single nucleotide.

4. The DNA sequence of Claim 1 wherein the alteration to said native sequence comprises a deletion.

5. The DNA sequence of Claim 4 comprising a DNA sequence selected from the group consisting of SEQ ID NOS:9-12 and 21.

6. The DNA sequence of Claim 1 wherein the alteration to said native sequence comprises an insertion.

7. The DNA sequence of Claim 2 derived from an organism selected from the group consisting of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

8. A recombinant DNA vector comprising a DNA sequence encoding a thermostable DNA polymerase altered in sequence relative to the native sequence such that it exhibits altered DNA synthetic activity from that of the native DNA polymerase.

9. The recombinant DNA vector comprising a DNA sequence of Claim 8 wherein the alteration to said native sequence comprises a change in a single nucleotide.

10. The recombinant DNA vector comprising a DNA sequence of Claim 8 wherein the alteration to said native sequence comprises a deletion.

11. The recombinant DNA vector comprising a DNA sequence of Claim 8 derived from an organism selected from the group consisting of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

12. The recombinant DNA vector comprising a DNA sequence of Claim 11 comprising a DNA sequence selected from the group consisting of SEQ ID NOS:9-12 and 21.

13. A host cell transformed with the recombinant vector of Claim 8.

14. A thermostable DNA polymerase altered in amino acid sequence such that it exhibits altered DNA synthetic activity from that of the native DNA polymerase but retains substantially the same 5' nuclease activity of that of the native DNA polymerase.

15. The polymerase of Claim 14 wherein the altered polymerase exhibits reduced synthetic activity from that of the native DNA polymerase.

16. The polymerase of Claim 15 wherein the alteration to said native sequence comprises a change in an amino acid.

17. The polymerase of Claim 15 wherein the alteration to said native sequence comprises a deletion.

18. The polymerase of Claim 15 derived from an organism selected from the group consisting of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

19. The polymerase of Claim 18 comprising an amino acid sequence encoded by the nucleic acid sequences selected from the group consisting of SEQ ID NOS:9-12 and 21.

20. A method of detecting the presence of a specific target DNA molecule comprising:

a) providing:

i) a target nucleic acid,

ii) a first oligonucleotide complementary to a first portion of said target nucleic acid, and

iii) a second oligonucleotide, a region of which is complementary to a second portion of said target nucleic acid, said non-complementary region of said second oligonucleotide providing a single-stranded arm at its 5' end;

b) mixing said target nucleic acid, said first oligonucleotide and said second oligonucleotide under conditions wherein said first oligonucleotide and the 3' end of said second oligonucleotide are annealed to said target DNA sequence so as to create a first cleavage structure;

c) providing a cleavage means under conditions such that cleavage of said first cleavage structure occurs preferentially at a site located within said second oligonucleotide in a manner dependent upon the annealing of said first and second oligonucleotides on said target nucleic acid, thereby liberating the single-stranded arm of said second oligonucleotide generating a third oligonucleotide;

d) providing a first hairpin structure having a single-stranded 3' arm and a single-stranded 5' arm under conditions wherein said third oligonucleotide anneals to said single-stranded 3' arm of said first hairpin thereby creating a second cleavage structure;

e) providing conditions under which cleavage of said second cleavage structure occurs by said cleavage means liberating the single-stranded 5' arm of said second cleavage structure so as to create reaction products comprising a fourth oligonucleotide and a first cleaved hairpin detection molecule;

f) providing a second hairpin structure having a single-stranded 3' arm and a single-stranded 5' arm under conditions wherein said fourth oligonucleotide anneals to the single-stranded 3' arm of said second hairpin thereby creating a third cleavage structure;

g) providing conditions under which cleavage of said third cleavage structure occurs by said cleavage means liberating the single-stranded 5' arm of said third cleavage structure so as to create reaction products comprising a fifth oligonucleotide identical in sequence to said third oligonucleotide and a second cleaved hairpin detection molecule; and

h) detecting the presence of said first and second cleaved hairpin detection molecules.

21. The method of Claim 20 wherein steps d) through g) are repeated at least once.

22. The method of Claim 20 wherein said cleavage means comprises an altered thermostable DNA polymerase such that cleavage reactions occur in the absence of any significant polymerase activity.

23. The method of Claim 20 wherein the cleavage reactions of steps c), e) and g) will not occur absent the annealing of said first oligonucleotide, said third oligonucleotide and said fourth oligonucleotide, respectively.

24. The method of Claim 20 wherein the cleavage reaction of step c) occurs within the annealed portion of said second oligonucleotide.

25. The method of Claim 20 wherein the cleavage reaction of step c) occurs within the non-annealed portion of said second oligonucleotide.

26. A method of detecting the presence of a specific target nucleic acid molecule comprising:

a) providing:

i) a cleavage means,

ii) a target nucleic acid,

iii) a first oligonucleotide complementary to a first portion of said target nucleic acid,

iv) a first solid support having a second oligonucleotide, a region of which is complementary to a second portion of said target nucleic acid, said non-complementary region of said second oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising a first signal oligonucleotide,

v) a plurality of uncleaved second solid supports each having a third oligonucleotide, a region of which is complementary to said first signal oligonucleotide, the non-complementary region of said third oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising a second signal oligonucleotide, and

vi) a plurality of uncleaved third solid supports each having a fourth oligonucleotide, a region of which is complementary to said second signal oligonucleotide, the non-complementary region of said fourth oligonucleotide providing a single-stranded arm at its 5' end, a portion of said 5' arm comprising said first signal oligonucleotide;

b) mixing said cleavage means, said target nucleic acid, said first oligonucleotide and said second oligonucleotide under conditions wherein said first oligonucleotide and the 3' end of said second oligonucleotide are annealed to said target DNA sequence so as to create a

first cleavage structure and cleavage of said first cleavage structure results in the liberating of said first signal oligonucleotide;

c) reacting said liberated first signal oligonucleotide with one of said plurality of second solid supports under conditions such that said first signal oligonucleotide hybridizes to said complementary region of said third oligonucleotide to create a second cleavage structure and cleavage of said second cleavage structure results in the liberating of said second signal oligonucleotide and a cleaved second solid support;

d) reacting said liberated second signal oligonucleotide with one of said plurality of third solid supports under conditions such that said second signal oligonucleotide hybridizes to said complementary region of said fourth oligonucleotide to create a third cleavage structure and cleavage of said third cleavage structure results in the liberating of a second molecule of said first signal oligonucleotide and a cleaved third solid support; and

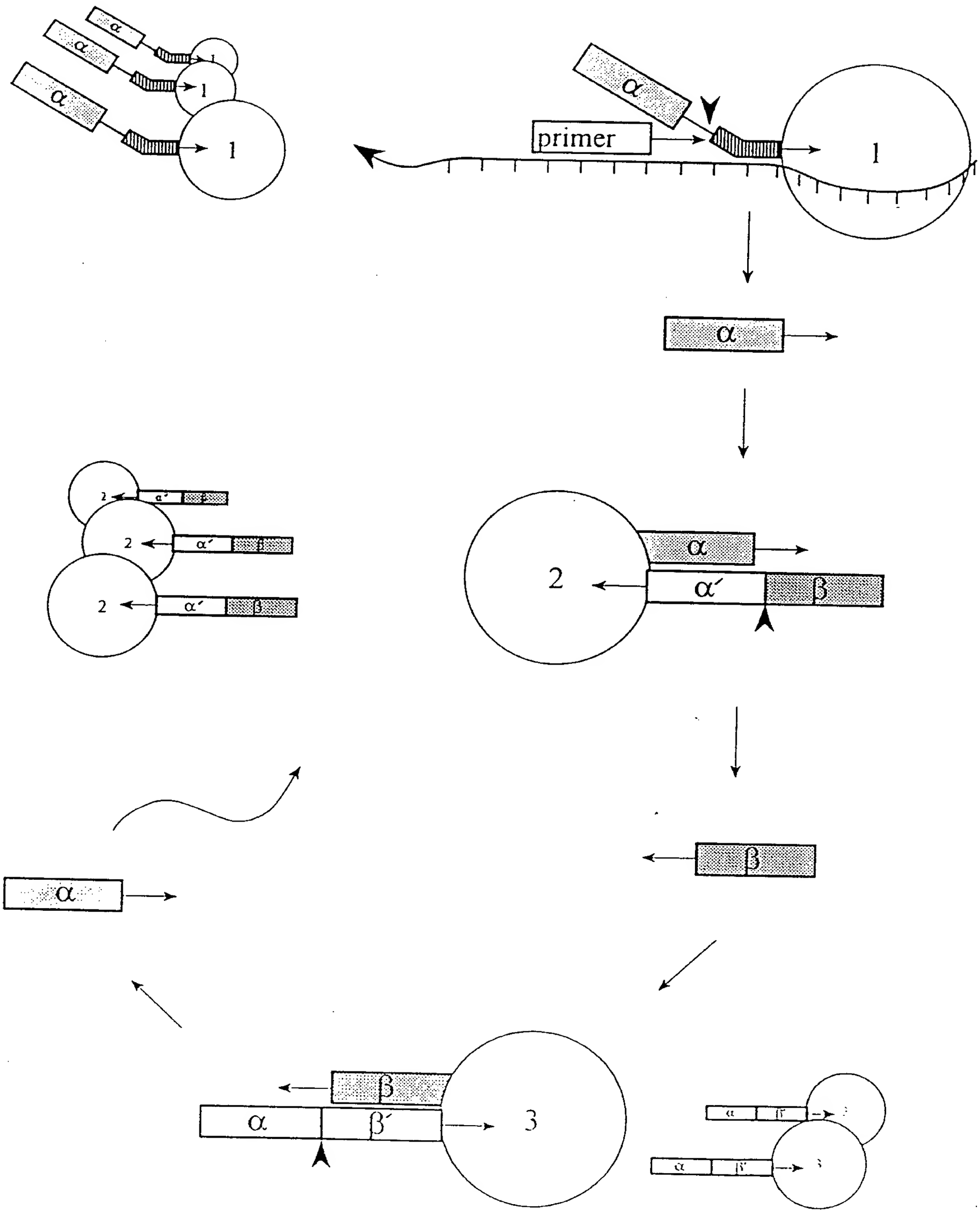
e) detecting the presence of said first and second signal oligonucleotides.

27. The method of Claim 26 wherein said cleavage means comprises a 5' nuclease derived from a thermostable DNA polymerase.

28. The method of Claim 27 wherein said thermostable DNA polymerase is derived from an organism selected from the group consisting of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

29. The method of Claim 28 wherein said 5' nuclease is encoded by a DNA sequence selected from the group consisting of SEQ ID NOS:11, 30 and 31.

FIGURE 1A



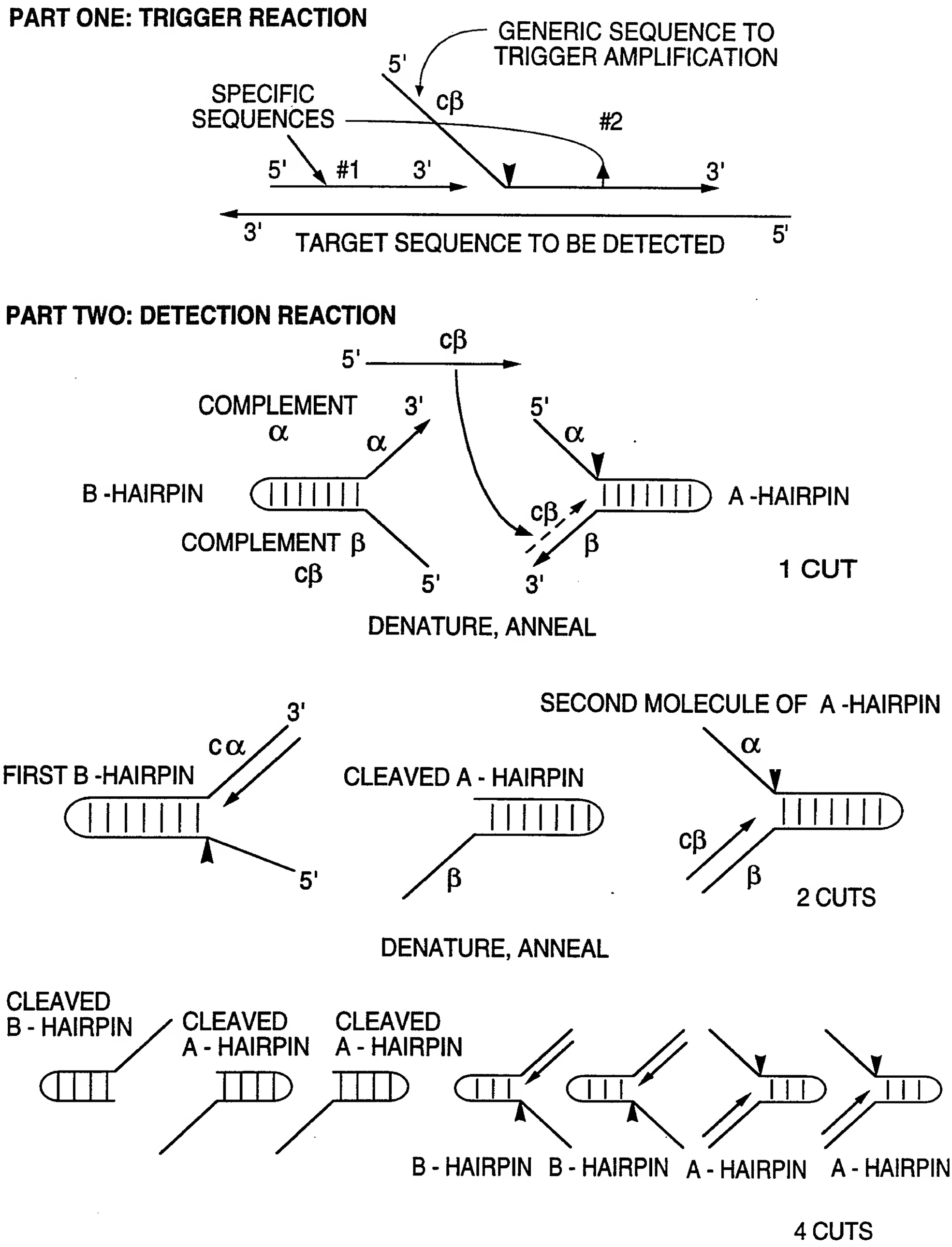


FIG. 1B

FIG. 2A

```
MAJORITY    ATGXXGGCGATGCTTCCCCTCTTTGAGCCCAAAGGCCGGGTCCTCCTGGTGGACGGCCACCACCTGGCCT

DNAPTAQ     ...AG..G......G.................G.....................................  70
DNAPTFL     .....................................C..G.....................................  67
DNAPTTH     ...GA............G........A.....................................................  70

MAJORITY    ACCGCACCTTCTTCGCCCTGAAGGCCTCACCACCAGCCGGGGCGAACCGGTGCAGGCGGTCTACGGCTT

DNAPTAQ     ............CA.................................G..G.....................  140
DNAPTFL     ............T.....C.....................C........C..T.....................  137
DNAPTTH     ..................................G.....................................  140

MAJORITY    CGCCAAGAGCCTCCTCAAGGCCCTGAAGGAGGACGGGGACXXGCGGTGXTCGTGGTCTTTGACGCCAAG

DNAPTAQ     ........................C.....................A.....................  207
DNAPTFL     ......A.....................................GT..T.....................  204
DNAPTTH     ..............................................T..AA...C..CT.....................  210

MAJORITY    GCCCCCTCCTTCGGCCACGAGGCCTACGAGGCCTACAAGGCGGGCCGGGCCCCCACCCCGGAGGACTTTC

DNAPTAQ     ..........................G..GG.....................G.....................  277
DNAPTFL     ..........................................................................  274
DNAPTTH     ..................................GA.......G.....C........C.  280

MAJORITY    CCCGGCAGCTCGCCCTCATCAAGGAGCTGGTGGACCTCCTGGGGCTTGCGCGCCTCGAGGTCCCCGGCTA

DNAPTAQ     .......A.....................................G.....................G.....  347
DNAPTFL     .........G.....................T.............A..C....T...G..G.....T.......T  344
DNAPTTH     ..................................T..A.C.....................  350
```

FIG. 2B

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MAJORITY    CGAGGCGGACGACGTXCTGGCCACCCTGGCCAAGAAGGCGGAAAAGGAGGGGTACGAGGTGCGCATCCTC

DNAPTAQ     ................C.......G.............................C........C.........  417
DNAPTFL     T...............G.................CG.................................  414
DNAPTTH     ................T..C.....................................................  420

MAJORITY    ACCGCCGACCGCGACCTCTACCAGCTCCTTTCCGACCGCATCGCCGTCCTCCACCCCGAGGGGTACCTCA

DNAPTAQ     .........AAA.....T.........................CA.............................  487
DNAPTFL     ..T.............................G..G.........A..........T...........G.  484
DNAPTTH     ......................A...G.C.........G.....................CC......  490

MAJORITY    TCACCCCGGCGTGGCTTTGGGAGAAGTACGGCCTGAGGCCGGAGCAGTGGGTGGACTACCGGGCCCTGGC

DNAPTAQ     ..........C...........A.................C..C.......CC................A.  557
DNAPTFL     ....................AC..............C.C.....................................  554
DNAPTTH     .........A.........................C.....................T...C.....C.T  560

MAJORITY    GGGGGACCCCTCCGACAACCTCCCCGGGGTCAAGGGCATCGGGGAGAAGACCGCCCXGAAGCTCCTCXAG

DNAPTAQ     C......GAG...........T.............................G..GAG......T..GG..  627
DNAPTFL     ............G..T...A.........G.........................A..G....A..CGC  624
DNAPTTH     ..................................................TC.........A..  630

MAJORITY    GAGTGGGGGAGCCTGGAAAACCTCCTCAAGAACCTGGACCGGGTGAAGCCCGC---CXTCGGGAGAAGA

DNAPTAQ     ....................GC.....................C.............---.A.....  694
DNAPTFL     ........................T..C..C.........A..........T.---.T.G.........C  691
DNAPTTH     .........A.....................................A.....A.AAAA.G.....  700
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FIG. 2C

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MAJORITY    TCCAGGCCCACATGGAXGACCTGAXGCTCTCCTGGGAGCTXTCCCAGGTGCGCACCGACCTGCCCCTGGA

DNAPTAQ     ...T.............C..T....A.............C..GG..A.......................... 764
DNAPTFL     .......GGG......G.C....GCC..T...C..A....T..........A...T.............. 761
DNAPTTH     ..A........C....A.....C.G........T......C....G..............C........ 770

MAJORITY    GGTGGACTTCGCCAAGXGGCGGGAGCCCGACCGGGAGGGGCTTAGGGCCTTTCTGGAGAGGCTGGAGTTT

DNAPTAQ     ................AA.....................A.............................T...... 834
DNAPTFL     ...........GG.G.C.C..CACA...A...T......T..GC....T...T.....C..T........ 831
DNAPTTH     .......C.....C..G.....................................C.................C 840

MAJORITY    GGCAGCCTCCTCCACGAGTTCGGCCTCCTGGAGGGCCCCAAGGCCCTGGAGGAGGCCCCCTGGCCCCCGC

DNAPTAQ     ..........................T.....AA..................................... 904
DNAPTFL     ..A.....................................G..G.....GGCA.....................T. 901
DNAPTTH     .....................................C....GCCC.......................... 910

MAJORITY    CGGAAGGGGCCTTCGTGGGCTTTGTCCTTTCCCGCCCCGAGCCCATGTGGGCCGAGCTTCTGGCCCTGGC

DNAPTAQ     ........................G.........AAG.................T.............. 974
DNAPTFL     ..........T..TT........TC.T.......T..................................... 971
DNAPTTH     ......................C.....C.....................G......AAA........ 980

MAJORITY    CGCCGCCAGGGAGGGCCGGGTCCACCGGGCACCAGACCCCTTTAXGGGCCTXAGGGACCTXAAGGAGGTG

DNAPTAQ     ...........G.....................C..C..G..T.A..AA.C...C........G.......C. 1044
DNAPTFL     T.GG..GT.......G..CC....T.......A.......C...G......G........T....G.... 1041
DNAPTTH     ....TG......C........G.........G..........GGC...G..A.A......C........C 1050
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FIG. 2D

```
MAJORITY  CGGGGXCTCCTCCCAAGGACCTGGCCGTTTTGGCCCTGAGGAGGCCTXCCGAGACCTCXTGCCCCGGGACC
DNAPTAQ   .....G..T.....AG.....C.....C.....A.....T.....CC.....C.....  1114
DNAPTR    .....AA.....G.....G.....C.....T.C..A.A.....  1111
DNAPTH    .....C.....C.....C.....TC.....G.....  1120

MAJORITY  ACCCCATGCTCCTCGCCCTACCTCCTGGACCCCCTCCAACACCACCCCCAGGCGCTACGG
DNAPTAQ   .....  1184
DNAPTR    .....T.....  1181
DNAPTH    .....G.....  1190

MAJORITY  GGGGGAGTGGACGGAGGAXGCGGGGGAGCGGGCCCTCCTXTCCGACAGGCTTCCXGAACCTXXXGGAG
DNAPTAQ   C.....G.....GCC.....GTG..G.  1254
DNAPTR    .....T.....A...C....AAA.....  1251
DNAPTH    .....C..C.CCC.C.....CCTTA..  1260

MAJORITY  CGCCTTGACGGGCAGGAGGCTCCTTTGGCTTTACCAGGAGGTGGAGAAGCCCCCTTTCCCGGGTCCTGC
DNAPTAQ   A.G.....GCT.....  1324
DNAPTR    .....A.....GT...  1321
DNAPTH    .....A.....C.....  1330

MAJORITY  CCCACATGGAGGCCACGGGGGTXCGGCTGGACGGTGGCCTACCTCCAGGCCCTXTXTCCTGGAGGTGGGGA
DNAPTAQ   .....G.....C..  1394
DNAPTR    .....C.....A..C  1391
DNAPTH    .....T.....C.T.....  1400

MAJORITY  CGGGGXCTCCTCCCAAGGACCTGGCCGTTTTGGCCCTGAGGAGGCCTXCCGAGACCTCXTGCCCCGGGACC
DNAPTAQ   .....G..T.....AG.....C.....C.....A.....T.....CC.....C.....  1114
DNAPTR    .....AA.....G.....G.....C.....T.C..A.A.....  1111
DNAPTH    .....C.....C.....C.....TC.....G.....  1120

MAJORITY  ACCCCATGCTCCTCGCCCTACCTCCTGGACCCCCTCCAACACCACCCCCAGGCGCTACGG
DNAPTAQ   .....  1184
DNAPTR    .....T.....  1181
DNAPTH    .....G.....  1190

MAJORITY  GGGGGAGTGGACGGAGGAXGCGGGGGAGCGGGCCCTCCTXTCCGACAGGCTTCCXGAACCTXXXGGAG
DNAPTAQ   C.....G.....GCC.....GTG..G.  1254
DNAPTR    .....T.....A...C....AAA.....  1251
DNAPTH    .....C..C.CCC.C.....CCTTA..  1260

MAJORITY  CGCCTTGACGGGCAGGAGGCTCCTTTGGCTTTACCAGGAGGTGGAGAAGCCCCCTTTCCCGGGTCCTGC
DNAPTAQ   A.G.....GCT.....  1324
DNAPTR    .....A.....GT...  1321
DNAPTH    .....C.....C.....  1330

MAJORITY  CCCACATGGAGGCCACGGGGGTXCGGCTGGACGGTGGCCTACCTCCAGGCCCTXTXTCCTGGAGGTGGGGA
DNAPTAQ   .....G..C.....C..  1394
DNAPTR    .....C.....A..C  1391
DNAPTH    .....A.....T.....  1400
```

FIG. 2E

```
MAJORITY    GGAGATCCGCCGCCTCGAGGAGGAGGTCTTCCGCCTGGCCGGCCACCCCTTCAACCTCAACTCCCGGGAC

DNAPTAQ     .......GC..........CC.....................................................  1464
DNAPTFL     ....G.G....AG..G.....................................................C...  1461
DNAPTTH     ..............................T....G.....................................  1470

MAJORITY    CAGCTGGAAAGGGTGCTCTTTGACGAGCTXGGGCTTCCCGCCATCGGCAAGACGGAGAAGACXGGCAAGC

DNAPTAQ     ..............C.............A.....................................C.......  1534
DNAPTFL     ........GC.....................G..C..G..T.....................G..G..A.  1531
DNAPTTH     ..............................TA..........T.G..G......C.A.....A.......  1540

MAJORITY    GCTCCACCAGCGCCGCCGTGCTGGAGGCCCTXCGXGAGGCCCACCCCATCGTGGAGAAGATCCTGCAGTA

DNAPTAQ     ....................C..........C..C.....................................  1604
DNAPTFL     .............T..................G..A.....................CCGC..........  1601
DNAPTTH     ................G..............A..G.....................................C...C.  1610

MAJORITY    CCGGGAGCTCACCAAGCTCAAGAACACCTACATXGACCCCCTGCCXGXCCTCGTCCACCCCAGGACGGGC

DNAPTAQ     ....................G....G.........T......T....G.A....A.................  1674
DNAPTFL     ..............................A..........C.C...G..........A...C...  1671
DNAPTTH     ..............................G.G........C..AAG............G.........  1680

MAJORITY    CGCCTCCACACCCGCTTCAACCAGACGGCCACGGCCACGGGCAGGCTTAGTAGCTCCGACCCCAACCTGC

DNAPTAQ     ..................................................A..........T........C.  1744
DNAPTFL     ..G.............................C..............TCC.....................  1741
DNAPTTH     ..................................................G.....................  1750
```

FIG. 2F

```
MAJORITY   AGAACATCCCCGTCCGCACCCCXCTGGGCCAGAGGATCCGCCGGGCCTTCGTGGCCGAGGAGGGXTGGGT

DNAPTAQ    .....G..T..G.....A.C.....G...C.  1814
DNAPTFL    .....G.....T.....C.C.....A.....C.....  1811
DNAPTTH    .....CT.....C...T....C  1820

MAJORITY   GTTGGTGGCCCTGGACTATAGCCAGATAGAGCTCCGGGTCCTGGCCCACCTCTCCGGGGACGAGAACCTG

DNAPTAQ    A.....A....G.....C.....  1884
DNAPTFL    .C.....T.T.....C.....T.....T.....  1881
DNAPTTH    .....C.....C.....A.....  1890

MAJORITY   ATCCGGGTCTTCCAGGAGGGGAGGGACATCCACACCCAGACCGCCAGCTGGATGTTCGGCGTCCCCCCGG

DNAPTAQ    .....C.....GG.....G..  1954
DNAPTFL    .....T.....TT....C.  1951
DNAPTTH    ...A.....A.....A.....  1960

MAJORITY   AGGCCGTGGACCCCCTGATGCGCCGGGCGGCCAAGACCATCAACTTCGGGGTCCTCTACGGCATGTCCGC

DNAPTAQ    .....G..  2024
DNAPTFL    .A.GG..A....T.....G.....  2021
DNAPTTH    .....GG.G.....C.....  2030

MAJORITY   CCACCGCCTCTCCCAGGAGCTTGCCATCCCCTACGAGGAGGCGGTGGCCTTCATTGAGCGCTACTTCCAG

DNAPTAQ    .....A.....T.....CCA.....T...  2094
DNAPTFL    .....GG.....T.....  2091
DNAPTTH    ...TA.G.....T..A.....A  2100
```

FIG. 2G

```
MAJORITY   AGCTTCCCCAAGGTGCGGGCCTGGATTGAGAAGACCCTGGAGGAGGGCAGGAGGCGGGGGTACGTGGAGA

DNAPTAQ    ..................................................................... 2164
DNAPTFL    ....A.....................GG.....C.........C.CC.........T..... 2161
DNAPTTH    .........................A..A.................G....A......C........A. 2170

MAJORITY   CCCTCTTCGGCCGCCGGCGCTACGTGCCCGACCTCAACGCCCGGGTGAAGAGCGTGCGGGAGGCGGCGGA

DNAPTAQ    ................C.........A.....AG.G.....................................C.. 2234
DNAPTFL    ....................T.....................................C.......... 2231
DNAPTTH    .........AA.AA.....................................CA.....C..... 2240

MAJORITY   GCGCATGGCCTTCAACATGCCCGTCCAGGGCACCGCCGCCGACCTCATGAAGCTGGCCATGGTGAAGCTC

DNAPTAQ    .....................................................T.......... 2304
DNAPTFL    ..................G.....................................CG...T 2301
DNAPTTH    .....................................................C.......... 2310

MAJORITY   TTCCCCCGGCTXCAGGAAATGGGGGCCAGGATGCTCCTXCAGGTCCACGACGAGCTGGTCCTCGAGGCCC

DNAPTAQ    ......A....GG.....................T..................................... 2374
DNAPTFL    ..........T.....C.....G.....TT.G.....G..................... 2371
DNAPTTH    ........C..C.G...G.........C.C.......C.................CC....G..... 2380

MAJORITY   CCAAAGAGCGGGCGGAGGXGGTGGCCGCTTTGGCCAAGGAGGTCATGGAGGGGGTCTATCCCCTGGCCGT

DNAPTAQ    .A.....A.........CC.....CGGC.....................G.......... 2444
DNAPTFL    ....G..C.........AG...A.....................GG.....CAG.. 2441
DNAPTTH    ..C...C.....C....A.....G.............C.....AA..C.........C..... 2450
```

FIG. 2H

```
MAJORITY    GCCCCTGGAGGTCAGGTCGGGGATGGGGAGGACTGGCTCTCGGCCAAGGA   2499
DNAPTAQ     ..........A.....................................   2496
DNAPTFL     ..........CC....................................   2505
DNAPTTH     ..........T.....................G...............
```

FIG. 3A

```
MAJORITY    MXAML PLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEPVQAVYGFAKSLLKALKEDG DAVXVVFDAK

TAQ PRO     .RG.....................H.....................................I..... 69
TFL PRO     .......................................................V.V..... 68
TTH PRO     .E.....................................................YK..F..... 70

MAJORITY    APSFRHEAYEAYKAGRAPTPEDFPRQLALIKELVDLLGLXRLEVPGYEADDVLATLAKKAEKEGYEVRIL

TAQ PRO     .........GG.....................................A.....S..... 139
TFL PRO     .....................................V.....F.....R..... 138
TTH PRO     .....................................FT..... 140

MAJORITY    TADRDLYQLLSDRIAVLHPEGYLITPAWLWEKYGLRPEQWVDYRALXGDPSDNLPGVKGIGEKTAXKLLX

TAQ PRO     ...K.....H.....D..A.....T..E.....R...E 209
TFL PRO     .....E...I.....Y.....A.....I.....QR.IR 208
TTH PRO     .....V...V.....H....E.....F...V.....L...K 210

MAJORITY    EWGSLENLLKNLDRVKP XXREKIXAHMEDLXLSXXLSXVRTDLPLEVDFAXRREPDREGLRAFLERLEF

TAQ PRO     ......A.....L..AI....L...D..K..WD.AK.....K.....R..... 278
TFL PRO     .....FQH..Q...SL...LQ.G..A.A..RK..Q.H.....GR..T.NL..... 277
TTH PRO     .....ENV....K..L...R..LE..R.....L.QG..... 280

MAJORITY    GSLLHEFGLLEXPKALEEAPWPPPEGAFVGFVLSRPEPMWAELLALAAARXGRVHRAXDPLXGLRDLKEV

TAQ PRO     .....S.....K.....D.....G.....PE.YKA.....A 348
TFL PRO     .....G...A.....L..SF.....G.WE..L...Q...R.....G. 347
TTH PRO     .....A.AP.....K....C.D.....A...A..K..... 350
```

FIG. 3B

```
MAJORITY    RGLLAKDLAVLALREGLDLXPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEDAGERALLSERLFXNLXX

TAQ PRO     .....S.....G.P.....................................E.....A.....A..WG  418
TFL PRO     ..I.................F.E.....................................A....QT.KE  417
TTH PRO     ...........S.....V.....................................AH.....HR..LK  420

MAJORITY    RLEGEERLLWLYXEVEKPLSRVLAHMEATGVRLDVAYLQALSLEVAEEIRRLEEEVFRLAGHPFNLNSRD

TAQ PRO     ...........R...R...A.................R.........A...A.................  488
TFL PRO     ..K.........E..........R.....................EA.V.Q.................  487
TTH PRO     ......K.....H.....................................L.................  490

MAJORITY    QLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKNTYIDPLPXLVHPRTG

TAQ PRO     .....................................................S.....D.I.....  558
TFL PRO     ..................................................DR.....A....K..  557
TTH PRO     .....R...L...Q.....................H.........V....S.....  560

MAJORITY    RLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFVAEEGWXLVALDYSQIELRVLAHLSGDENL

TAQ PRO     .....................................I.....L.....  628
TFL PRO     .....................................V..V.....  627
TTH PRO     .....................................A..A.....  630

MAJORITY    IRVFQEGRDIHTQTASWMFGVPPEAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAVAFIERYFQ

TAQ PRO     ...........E.........R.....................................Q.....  698
TFL PRO     .....................S..G.....................G..S.....  697
TTH PRO     .....K.....................V.....  700
```

FIG. 3C

```
MAJORITY  SFPKVRAWIEKFNMPVQGTAADLMKLAMVKL                                768
TAQ PRO   ..............................                                 768
TRL PRO   .Y............G.....R.                                          767
TTH PRO   ..............................                                 770

MAJORITY  FPRLXEMGARMLEEGRRGYVETLFGRRRYVPDLNARVKSVREAAERMA
TAQ PRO   ..............E...........
TRL PRO   ..............E...........
TTH PRO   ..............E...........

MAJORITY  LLQVHDELVLEAPKXRAEXVAALAKEVMEGVYPLAVPLEVEVGXGEDWLSAKEX      833
TAQ PRO   ....E...A..R..................W..Q....                      833
TRL PRO   ....Q.L.......................                            831
TTH PRO   ....R.........................                            835

MAJORITY  SFPKVRAWIEKFNMPVQGTAADLMKLAMVKL
TAQ PRO   ..............................
TRL PRO   .Y............G.....R.
TTH PRO   ..............................

MAJORITY  FPRLXEMGARMLEEGRRGYVETLFGRRRYVPDLNARVKSVREAAERMA
TAQ PRO   ....E.....................E...A..R..............
TRL PRO   ....Q.L...................D...R..............W..Q....
TTH PRO   ....R.....................L....OA...E..............A..KA.....

MAJORITY  LLQVHDELVLEAPKXRAEXVAALAKEVMEGVYPLAVPLEVEVGXGEDWLSAKEX
TAQ PRO   ....E...A..R..................W..Q....
TRL PRO   ....Q.L.......................
TTH PRO   ....R.........................
```

FIGURE 4

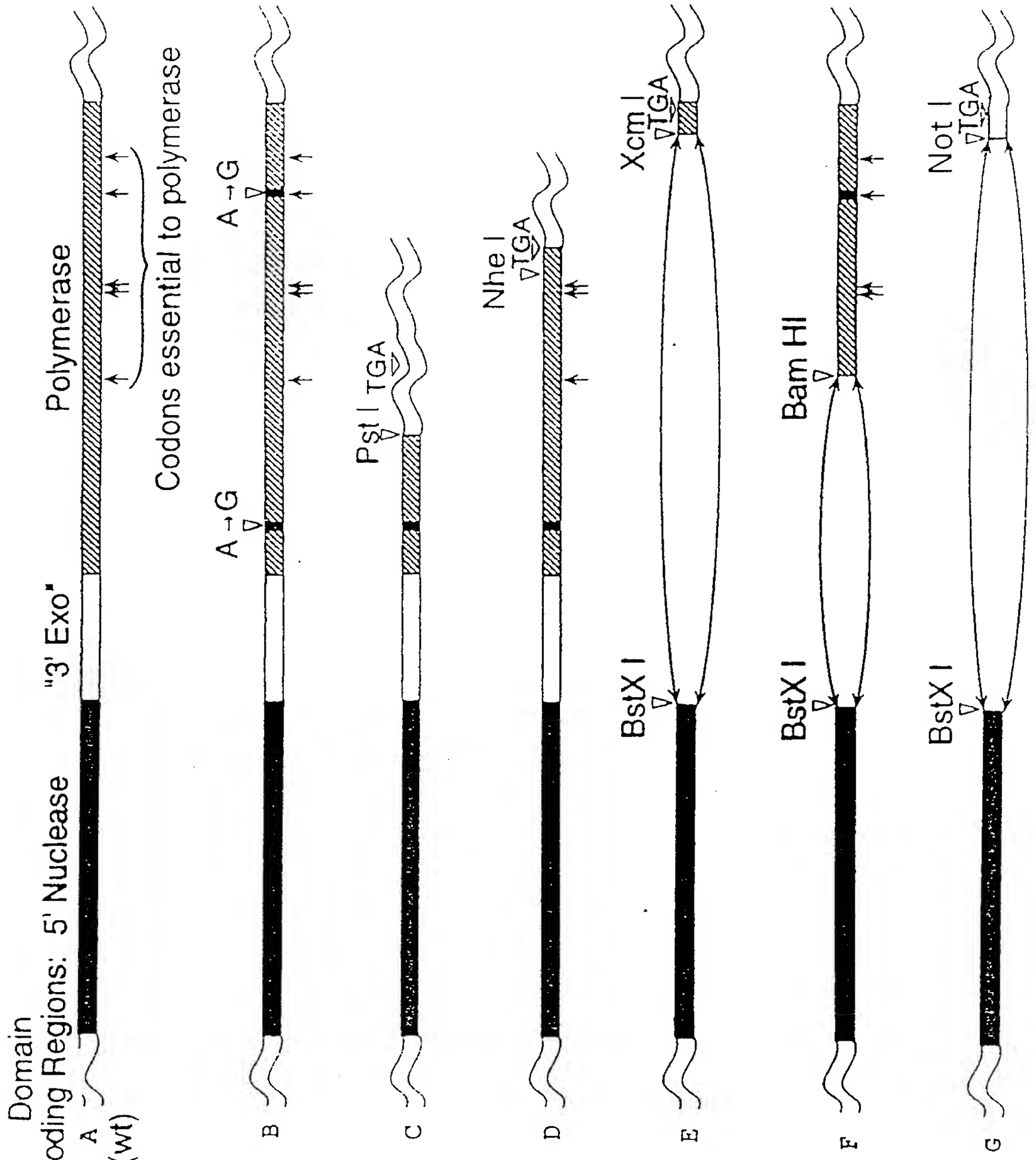


FIGURE 5

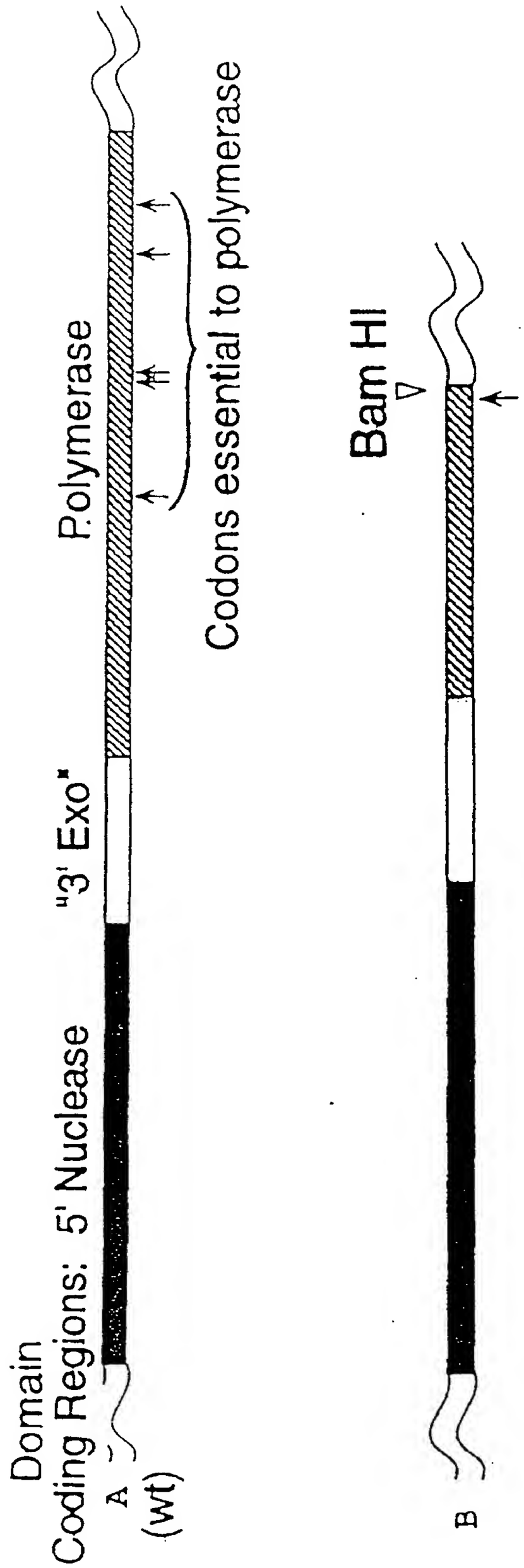
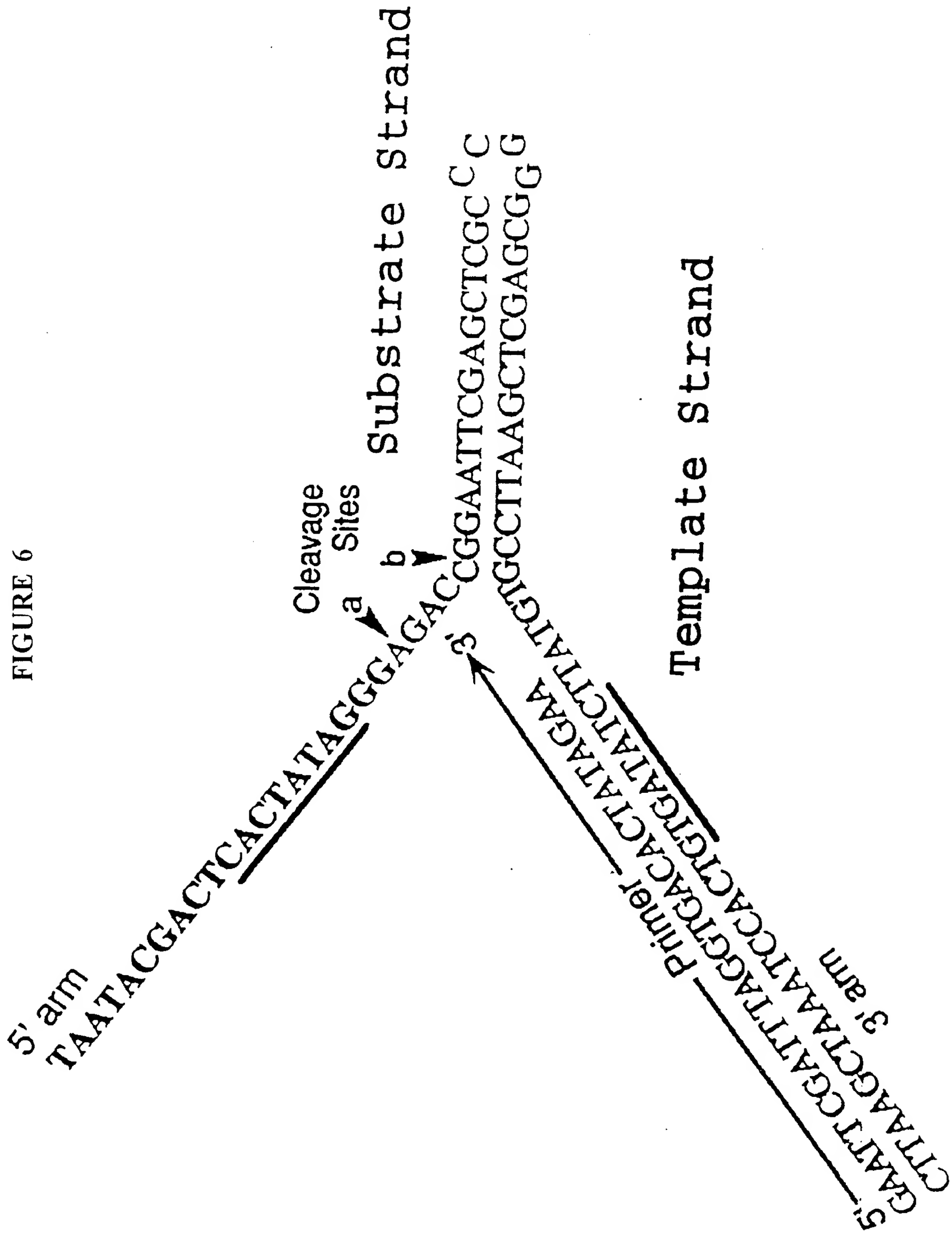


FIGURE 6


Substrate Strand
Cleavage Sites
a
b
Template Strand
5' arm
TAATACGACTCACTATAGGGAGAC
CGGAATTCGAGCTCGGC C
GCCTTAAGCTCGAGCCG G
CATTCGATTTAGGTGACACTATAGAA
CTTAAGCTAAATCCACTGTGATATCTT
Primer
3' arm

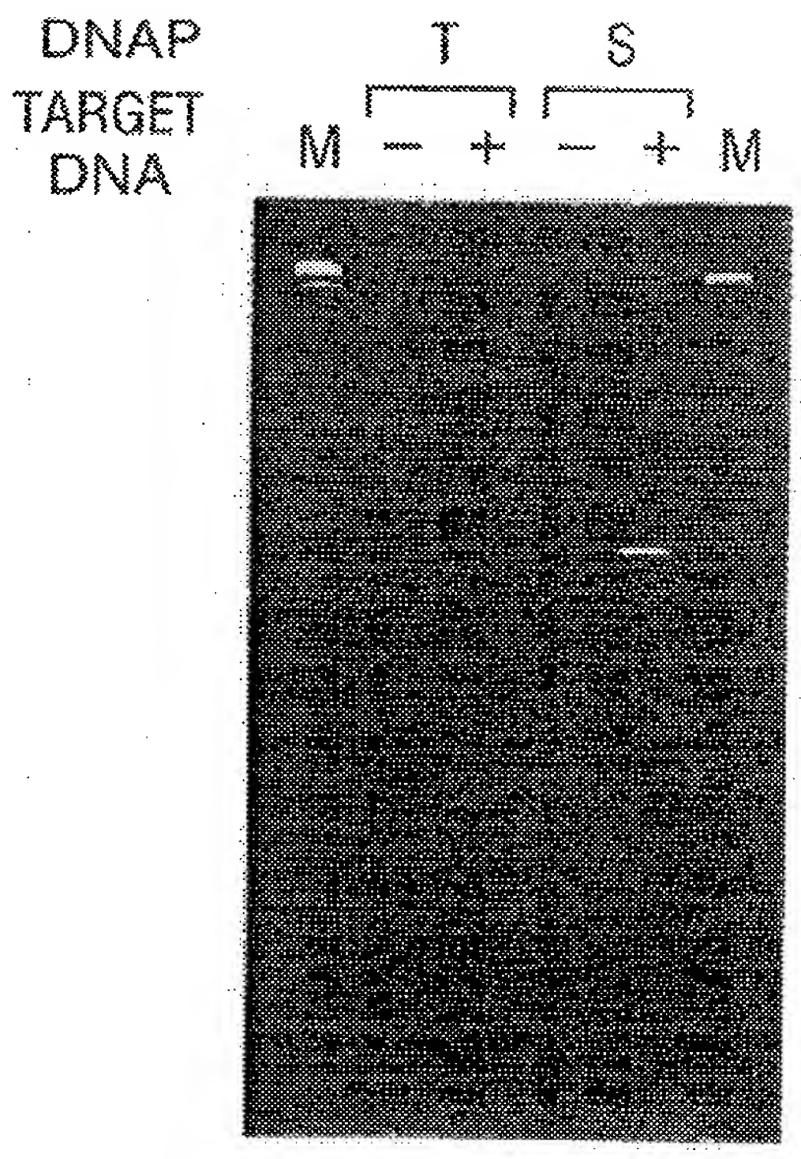


FIG. 7

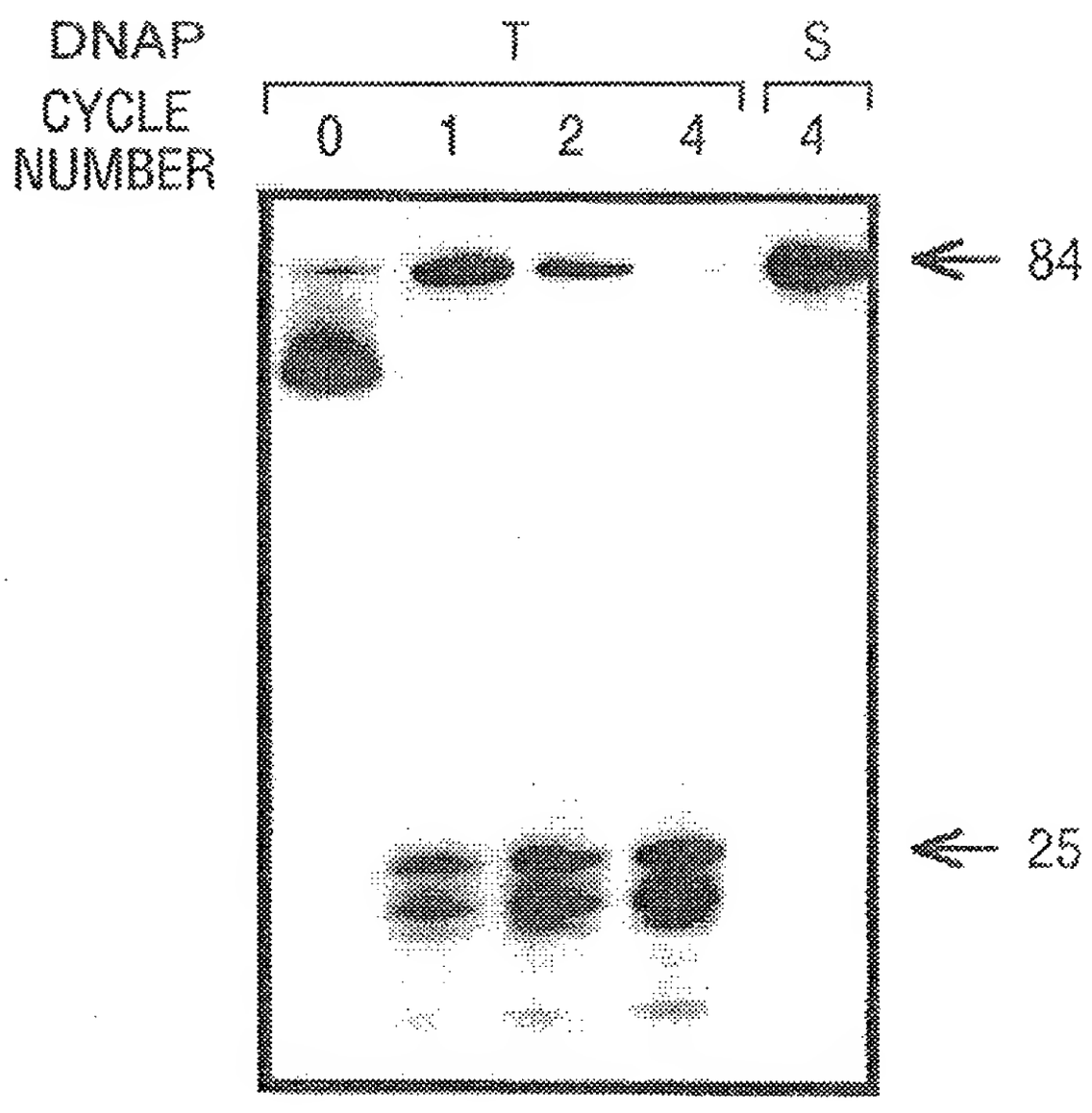


FIG. 8

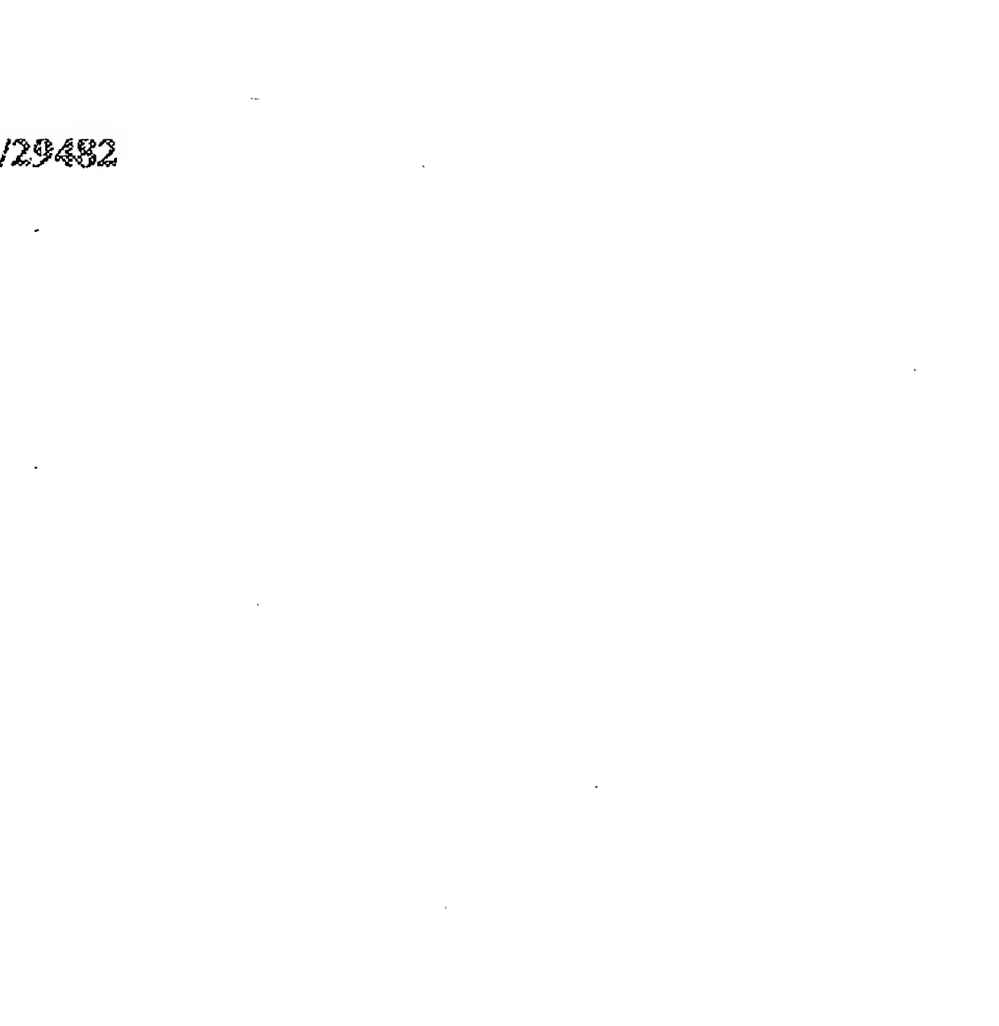


FIG. 9A

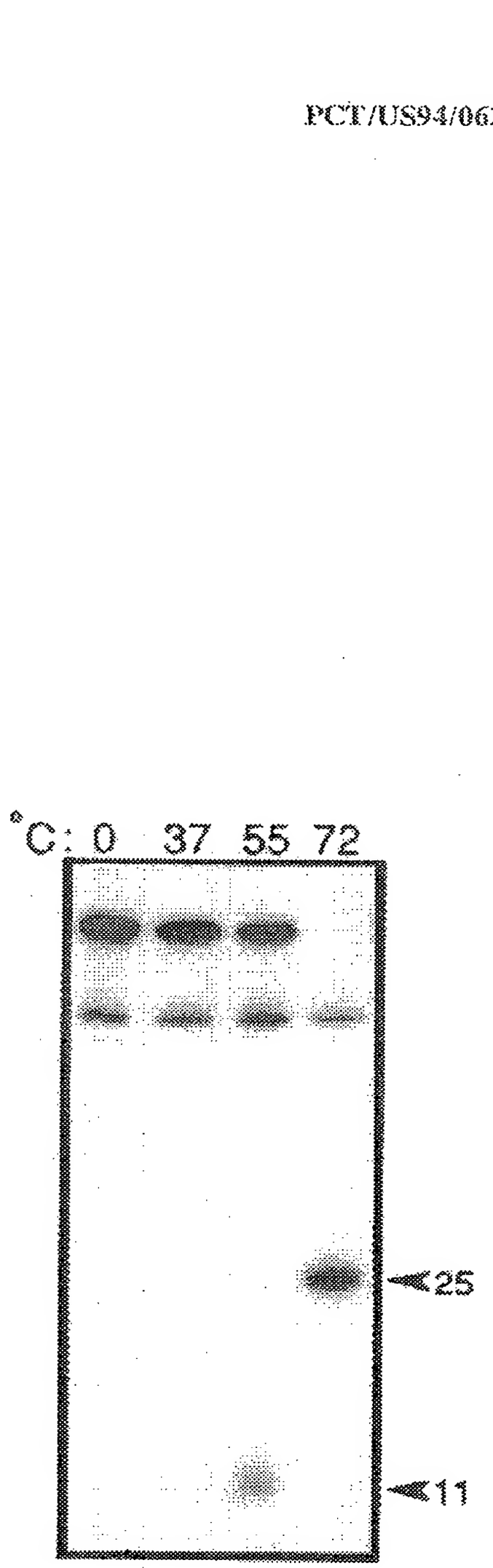


FIG. 9B

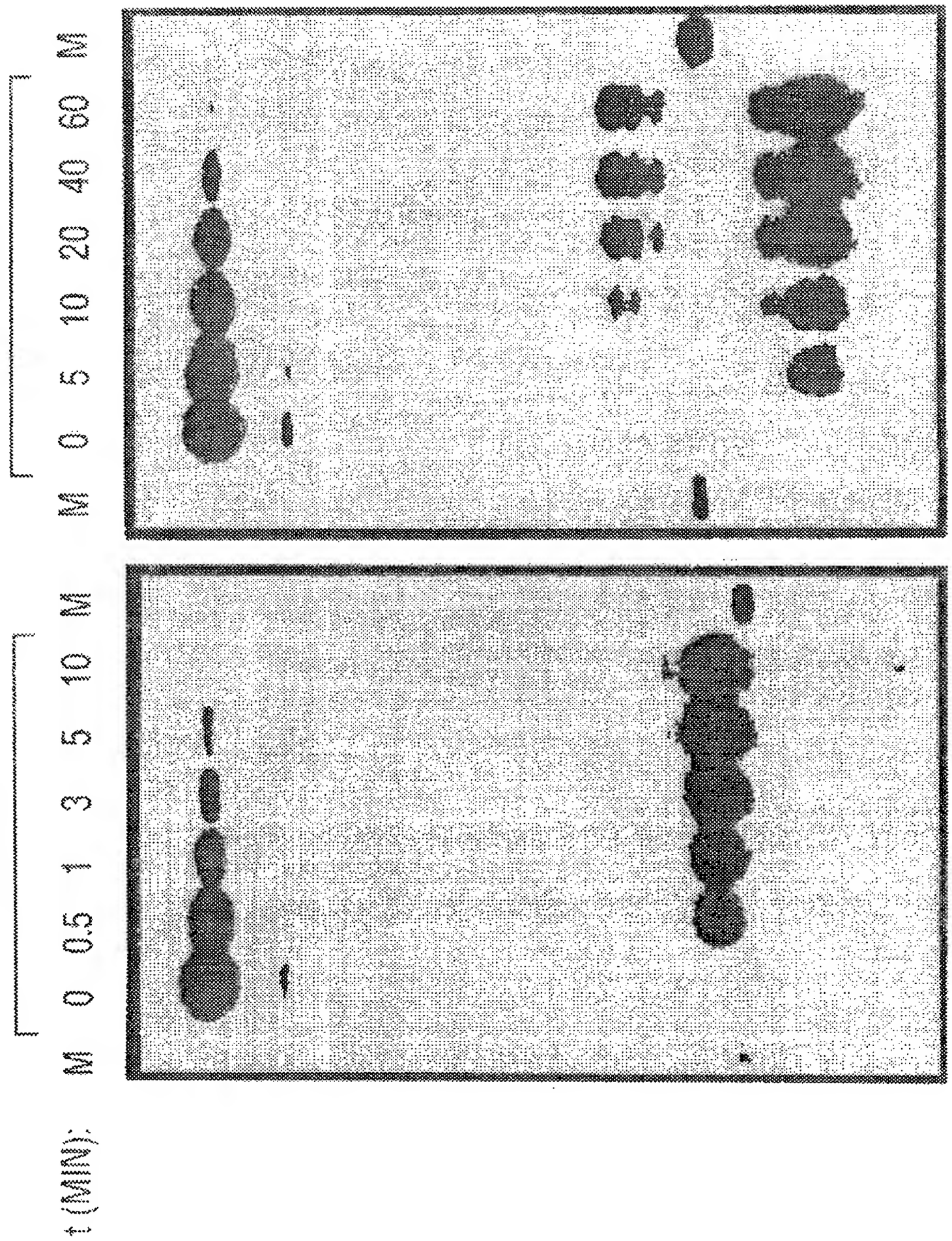


FIG. 10A FIG. 10B

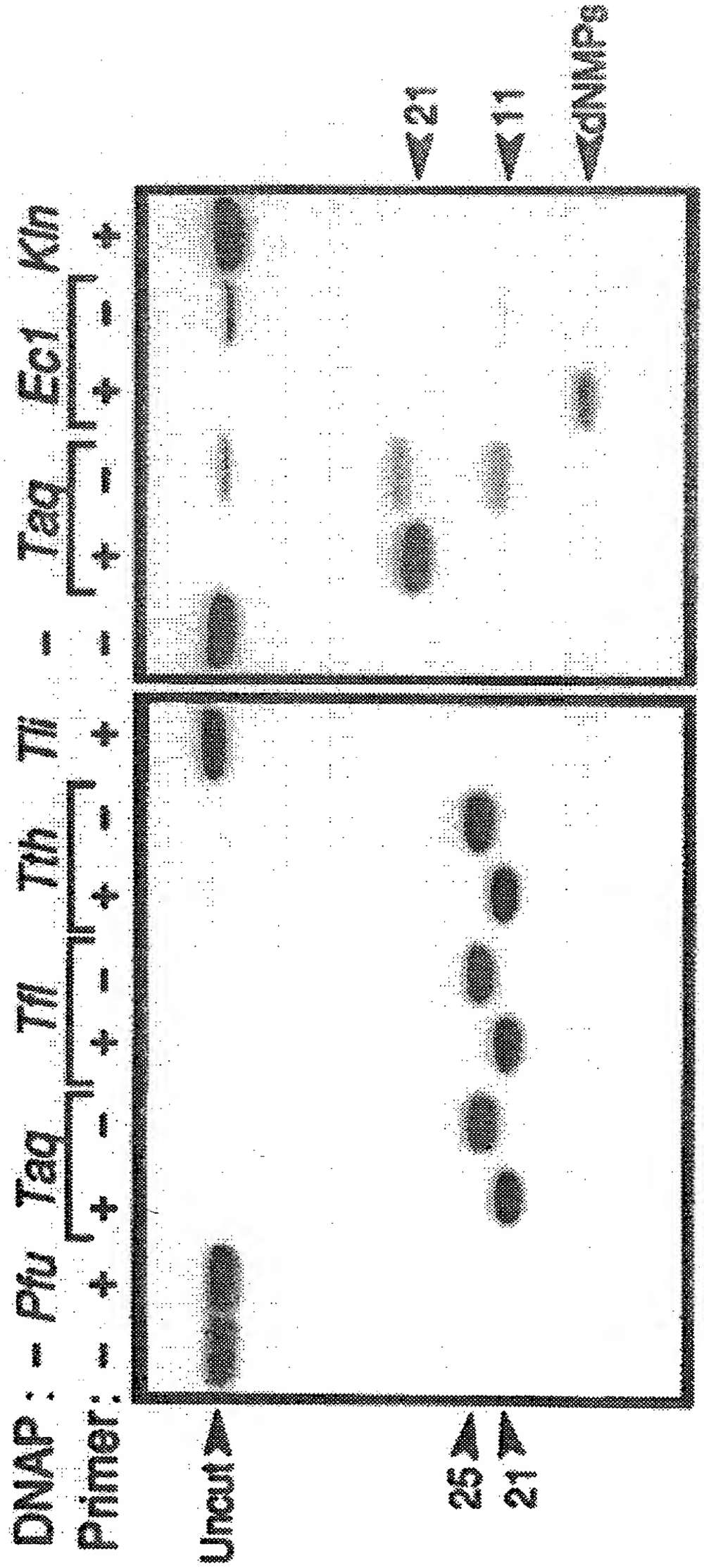


FIG. 11A FIG. 11B

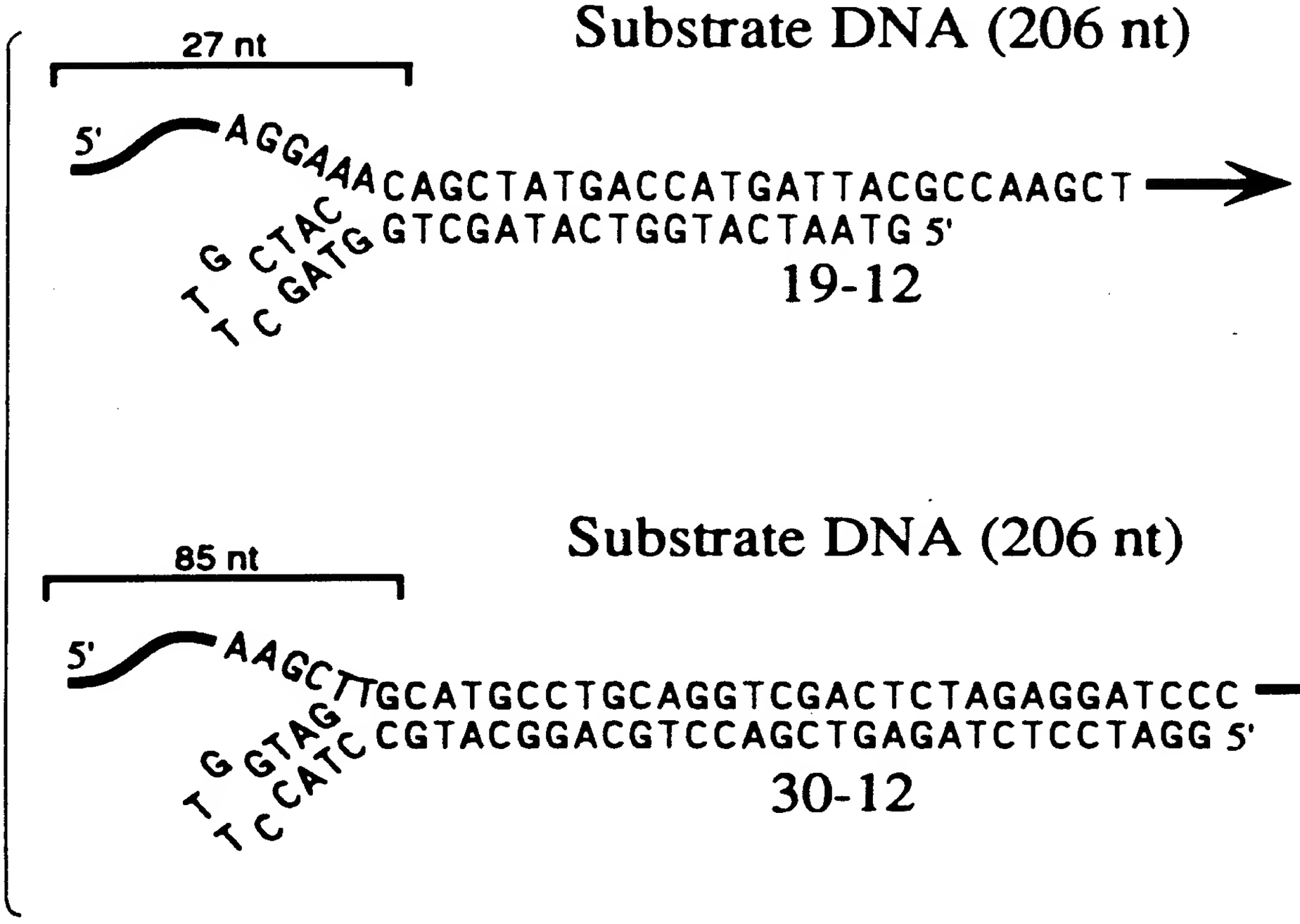


FIG. 12A

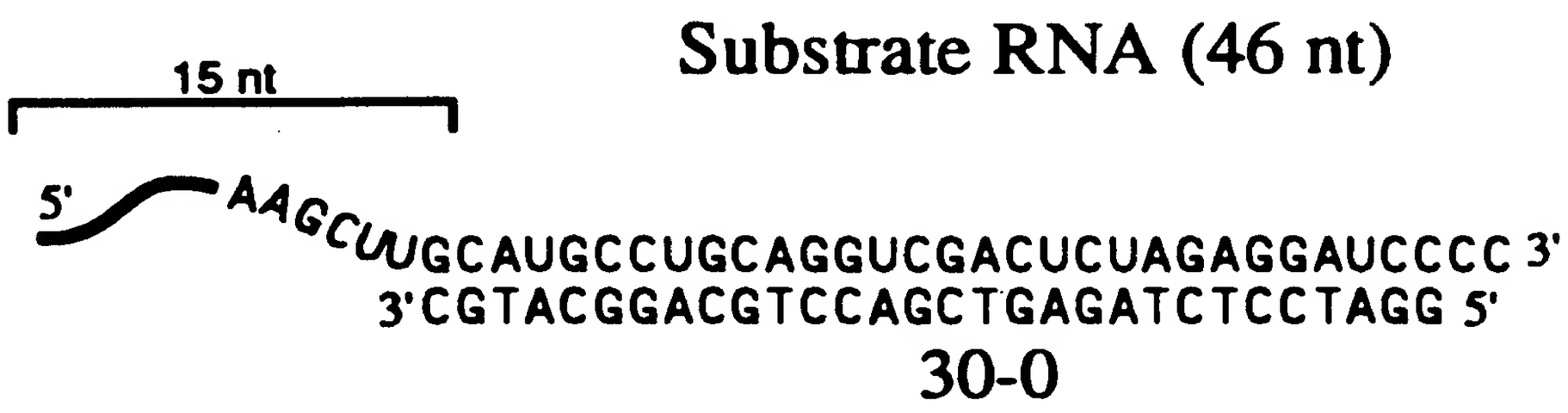


FIG. 13A

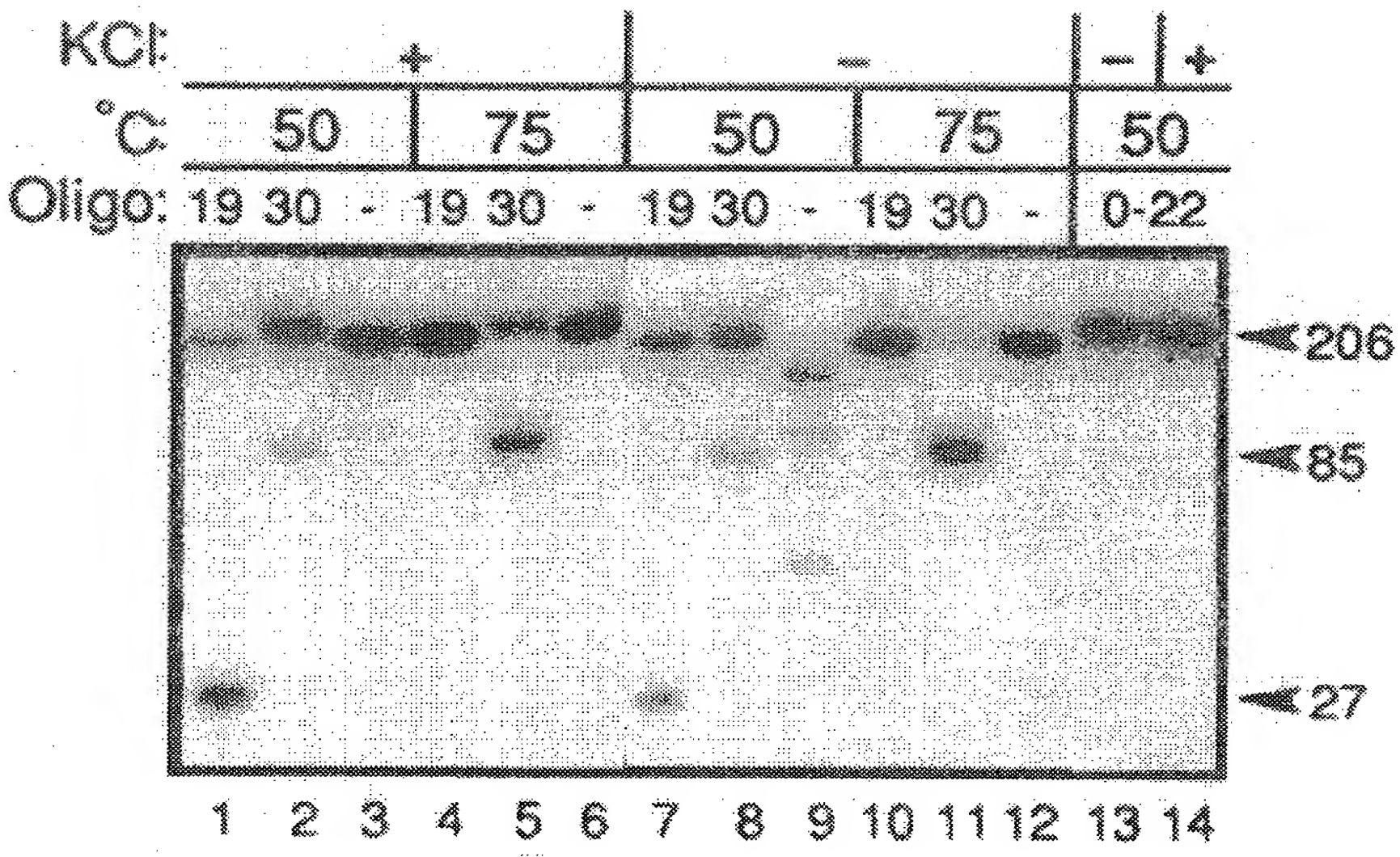


FIG. 12B

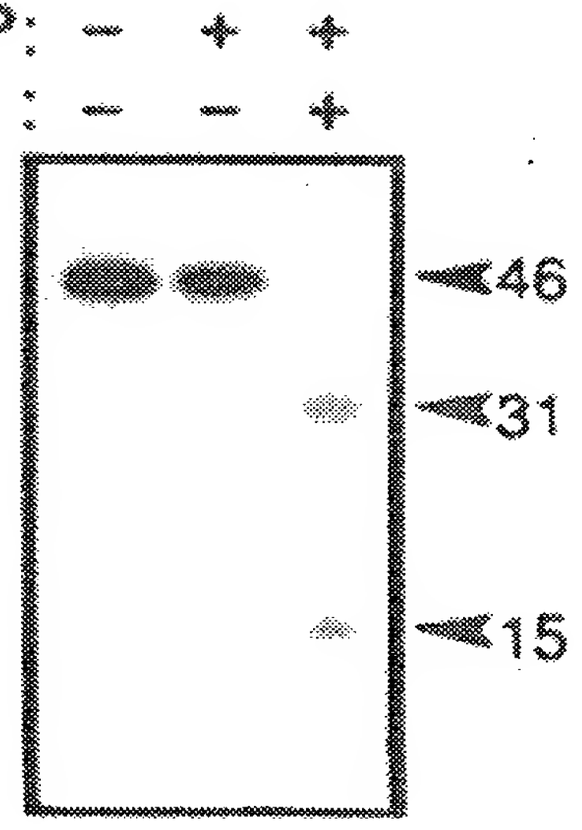
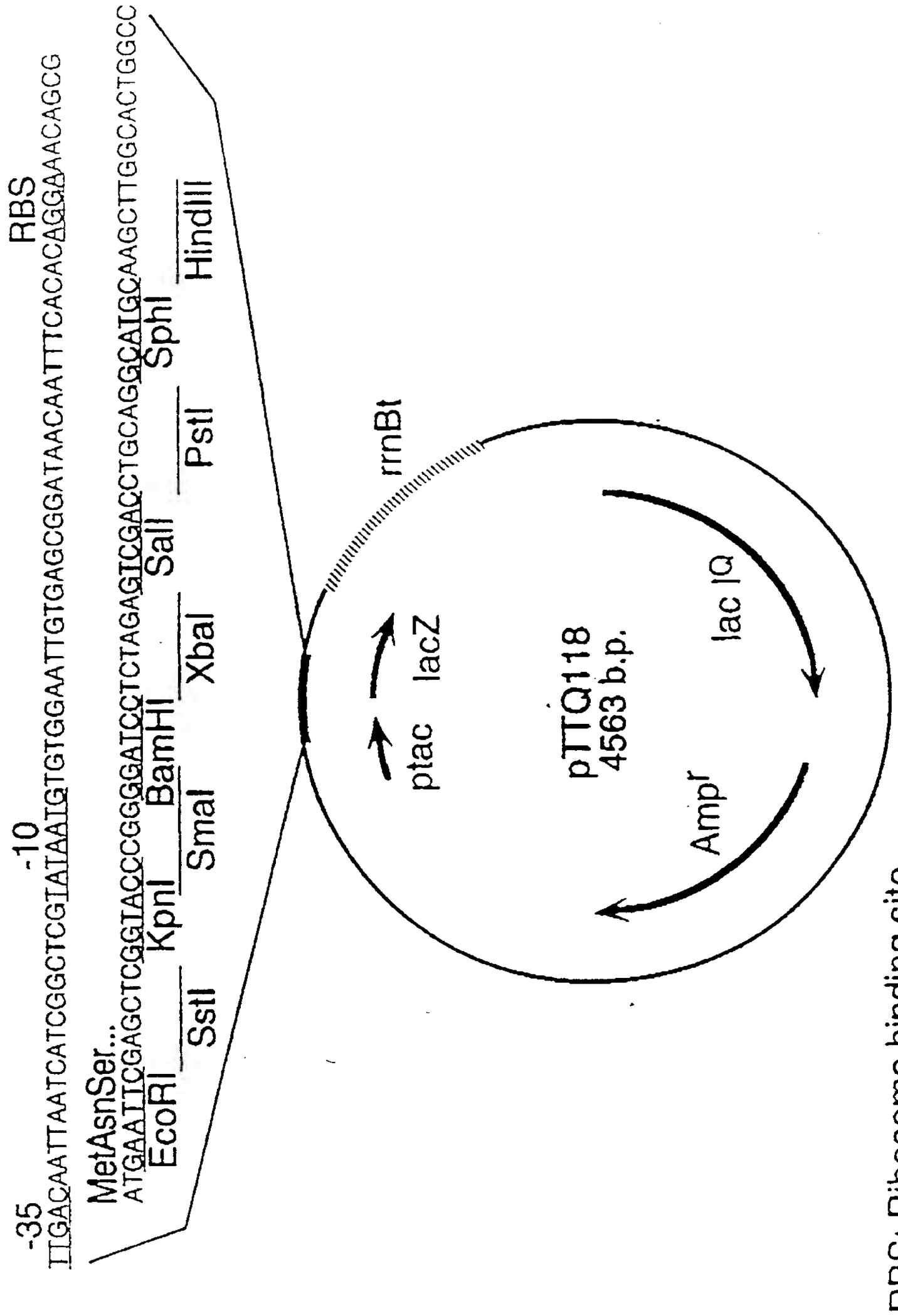


FIG. 13B

FIGURE 14



RBS: Ribosome binding site

ptac: Synthetic tac promoter

lac IQ: Lac repressor gene

lacZ: Beta-galactosidase alpha fragment

rrnBt: E. coli rrnB transcription terminator

FIGURE 15

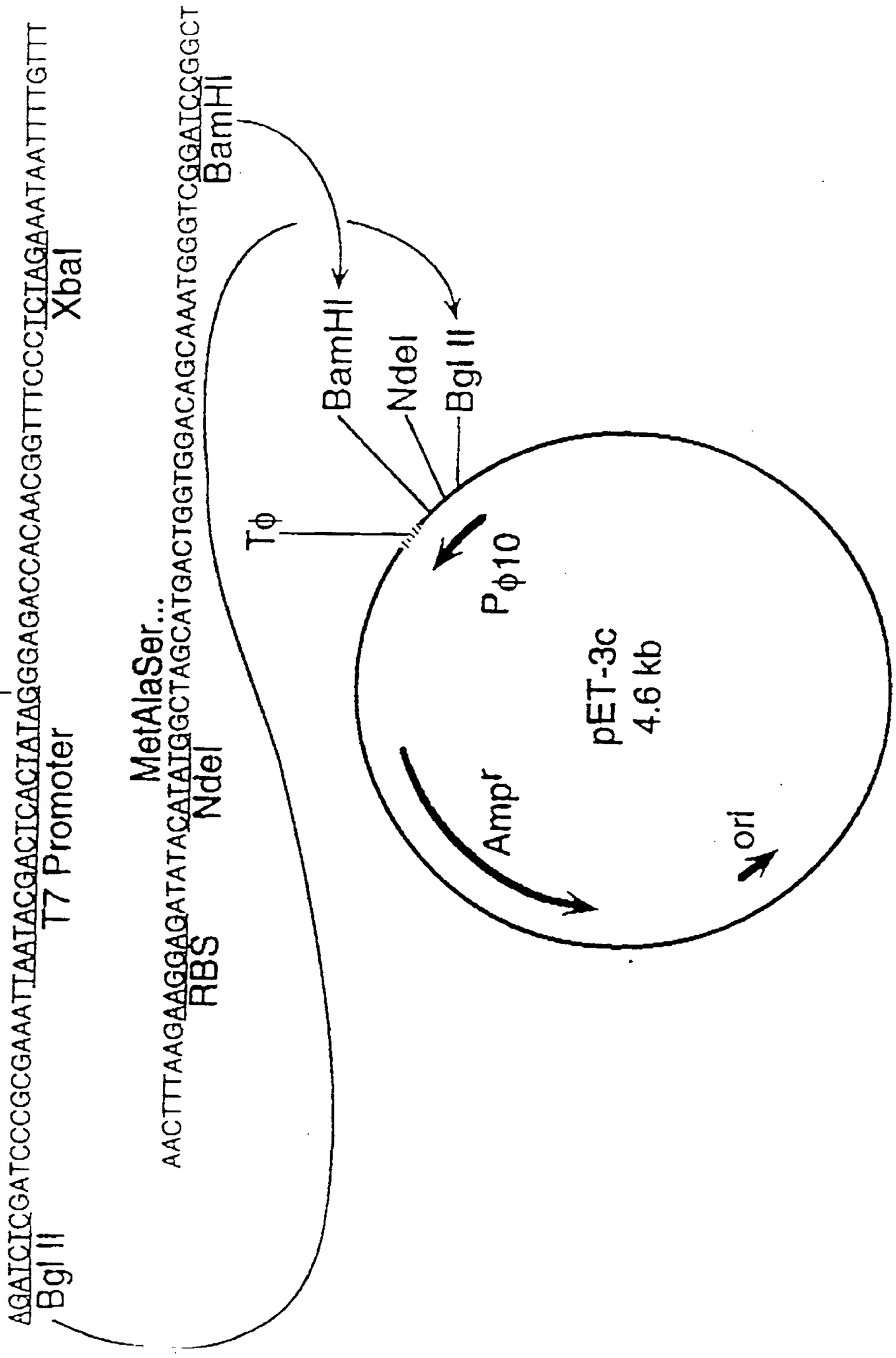
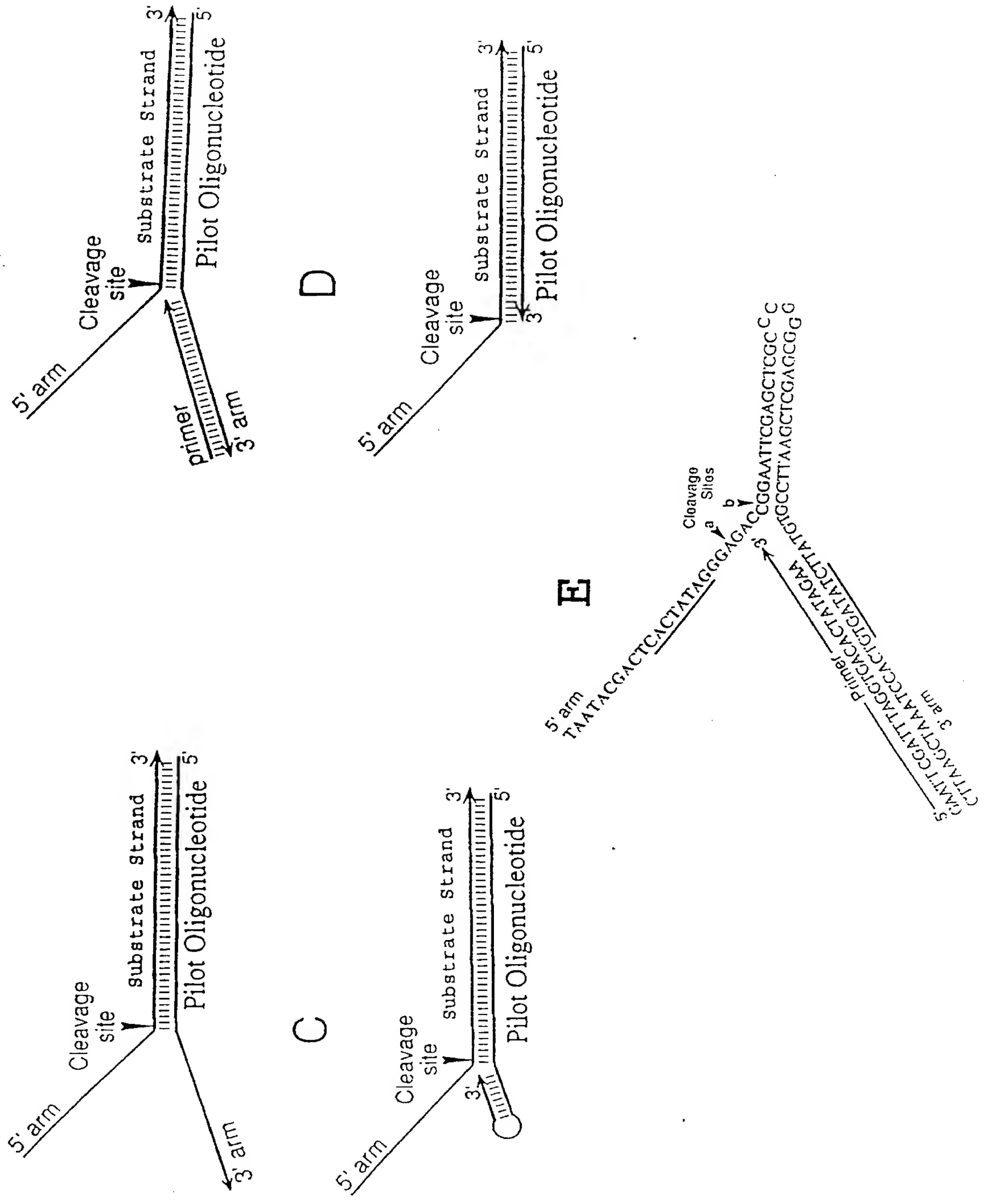


FIGURE 16


A
Cleavage site
Substrate Strand
Pilot Oligonucleotide
5' arm
3' arm
B
Cleavage site
Substrate Strand
Pilot Oligonucleotide
5' arm
primer
3' arm
C
Cleavage site
Substrate Strand
Pilot Oligonucleotide
5' arm
3'
D
Cleavage site
Substrate Strand
Pilot Oligonucleotide
5' arm
E
Cleavage Sites
a
b
5' arm
TAATACGACTCACTATAGGAGACCGGAATTCGAGCTCGGC C C
3'
ATTATGCTGAGTGATATCTTAAGCTCGAGCCGG G C
Primer
3' arm
5'

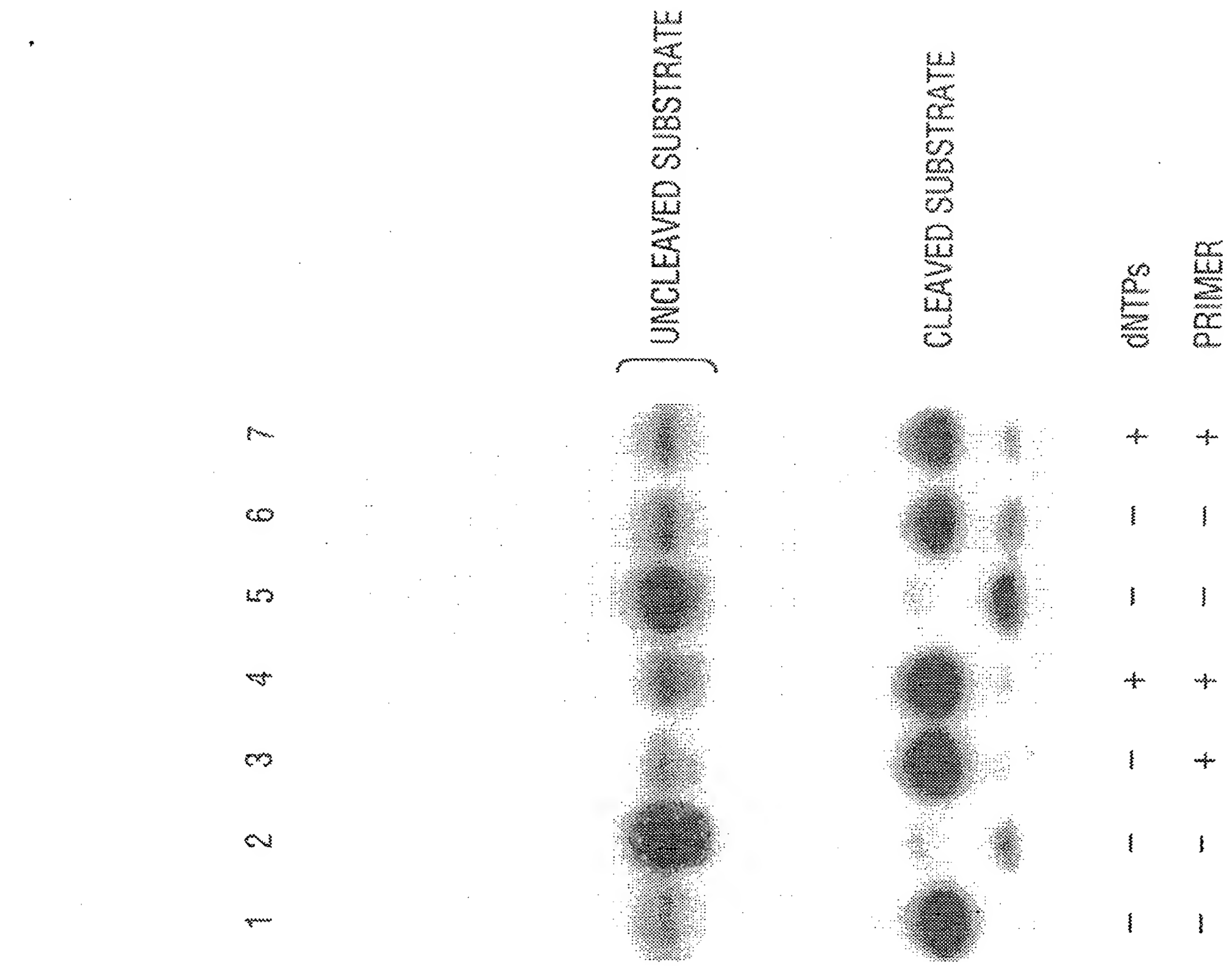

Taq
ENZYME
PRIMER
dNTPS
CLEAVED
UNCLEAVED SUBSTRATE
SUBSTRATE
1 2 3 4 5 6 7
4a
5b

FIG. 17

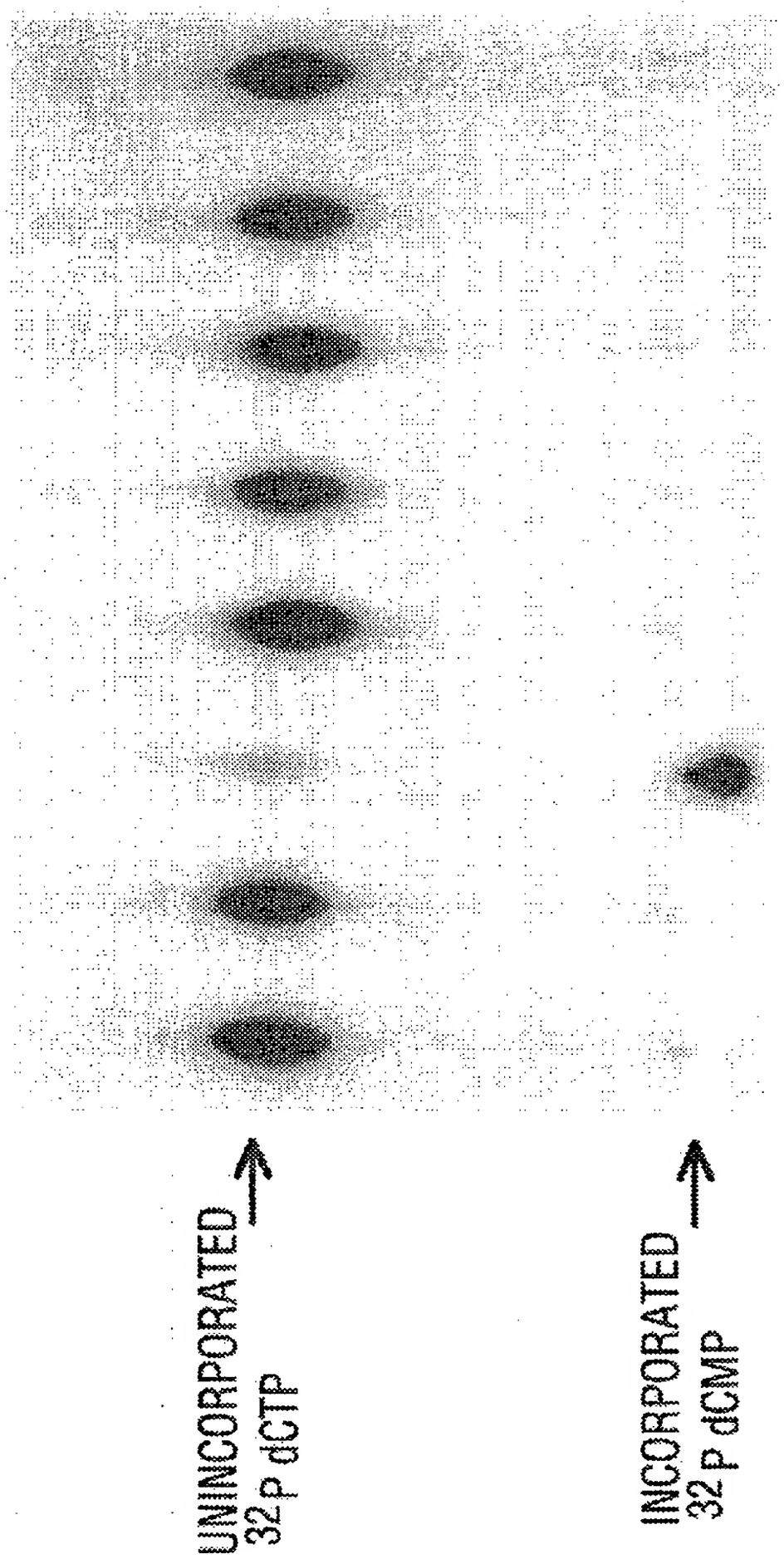

UNINCORPORATED
32 P dCTP
INCORPORATED
32 P dCMP
ENZYME
PRIMED M13
Taq – Taq 4b 4c 4d
– + + + +
4e + +
4f + +

FIG. 18

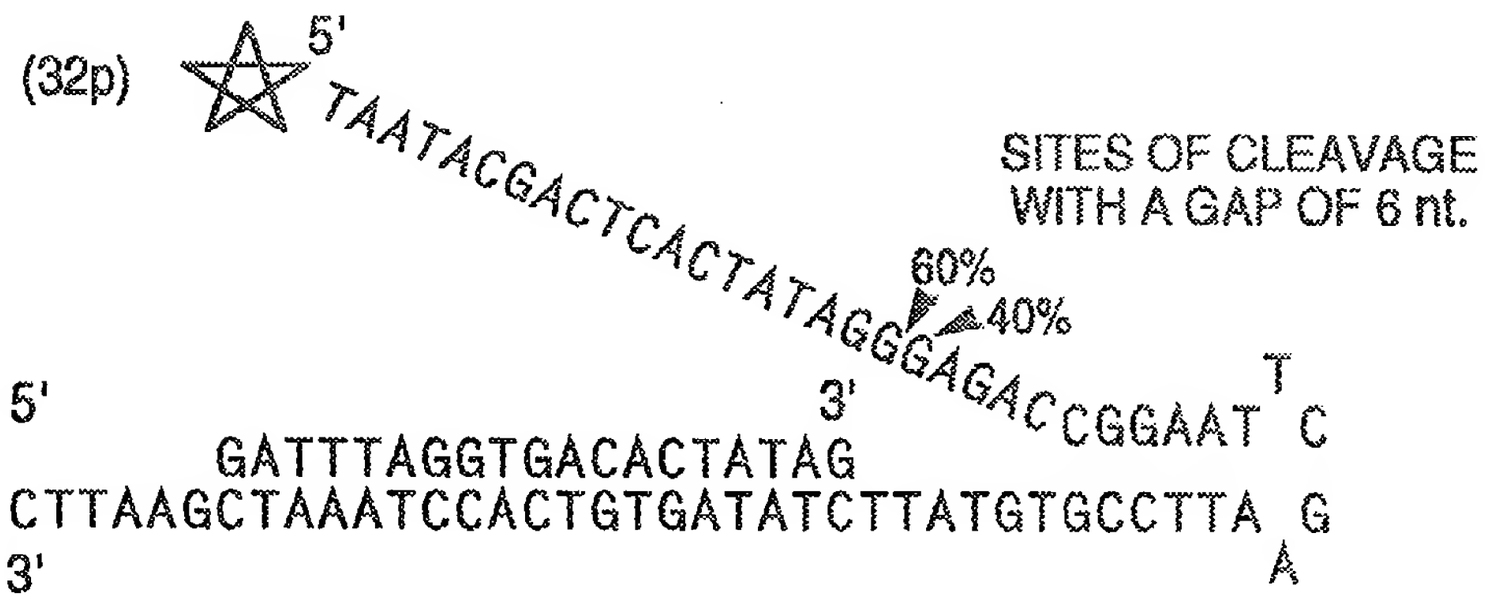

(32p)
5'
TAATACGACTCACTATAGGGAGACCGGAAT T C
SITES OF CLEAVAGE
WITH A GAP OF 6 nt.
60%
40%
5'
3'
GATTTAGGTGACACTATAG
CTTAAGCTAAATCCACTGTGATATCTTATGTGCCTTA G
3'
A

FIG. 19A

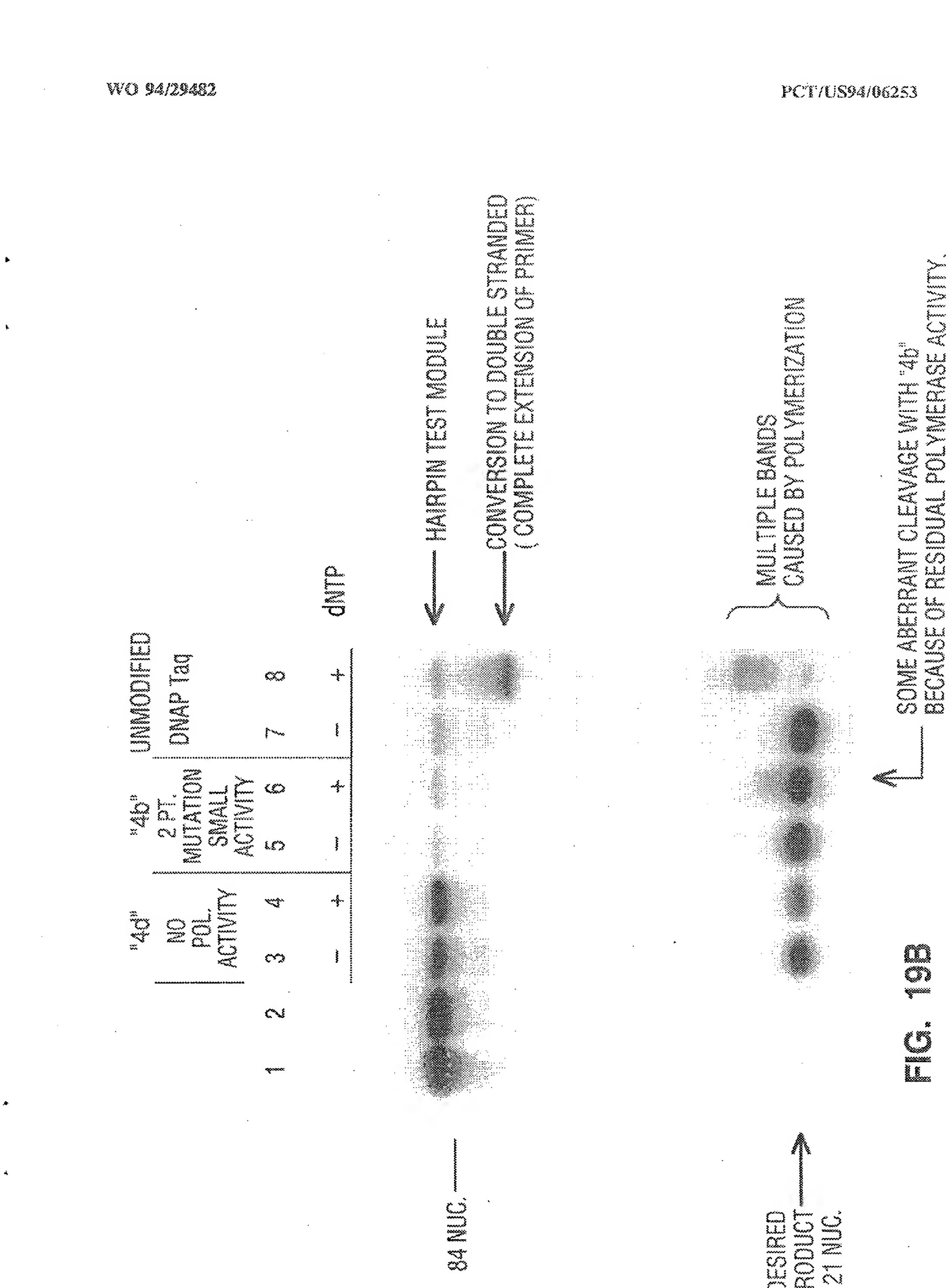

"4c" NO POL. ACTIVITY
"4b" 2 PT. MUTATION SMALL ACTIVITY
UNMODIFIED DNAP Taq
4 5 6 7 8
1 2 3
+ - + - +
dNTP
HAIRPIN TEMPLATE
CONVERSION TO DOUBLE STRANDED (COMPLETE EXTENSION OF PRIMER)
MULTIPLE BANDS CAUSED BY POLYMERIZATION
SOME ABERRANT CLEAVAGE BECAUSE OF RESIDUAL POLYMERASE ACTIVITY.
84 NUC.
DESIRED PRODUCT 21 NUC.

FIG. 19B

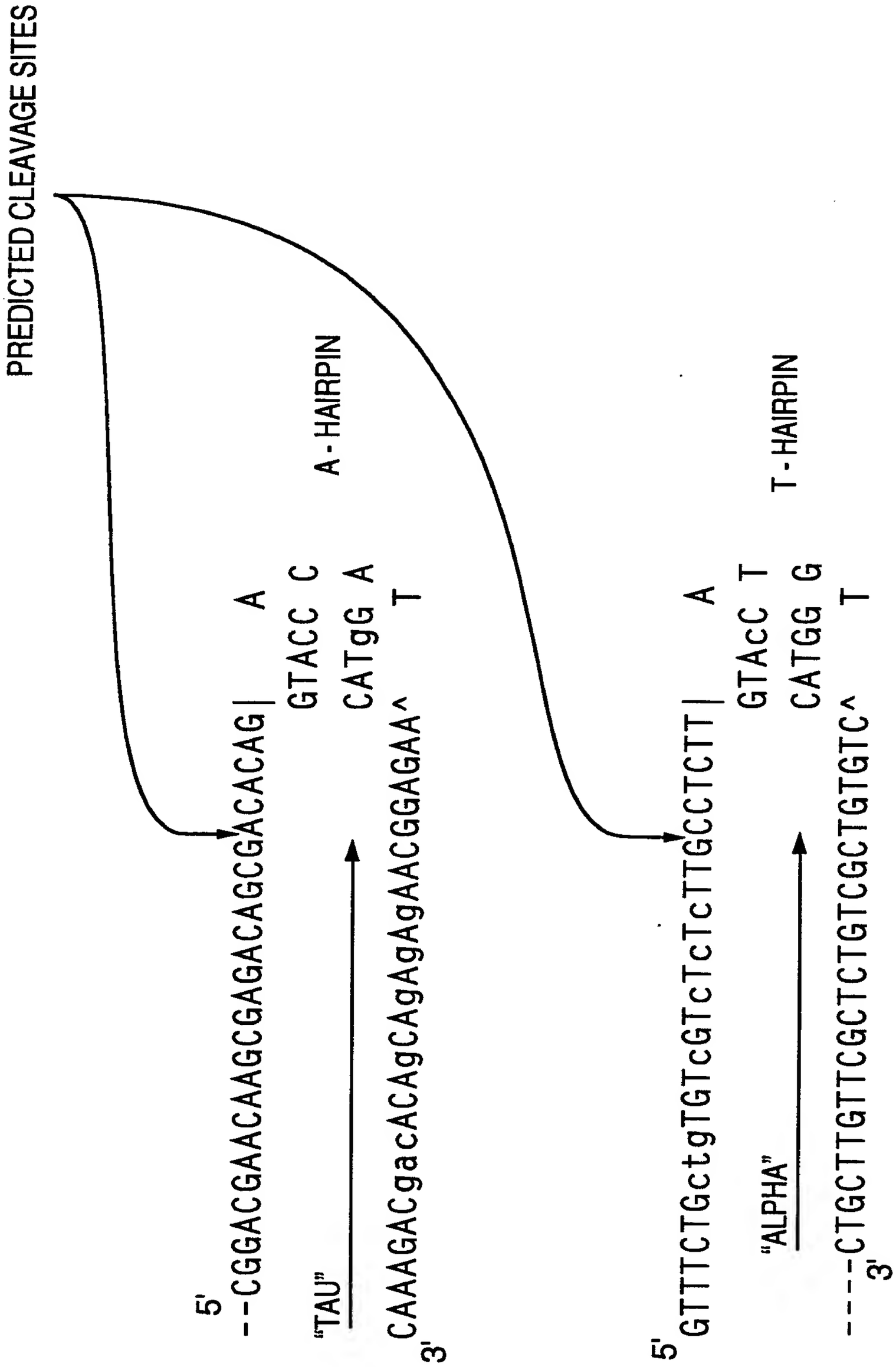

PREDICTED CLEAVAGE SITES
5'
--CGGACGAACAAGCGAGACACAG| A
"TAU"
3' CAAAGACgacACAgCAgAgAACGGAGAA^ T
A-HAIRPIN
5'
GTTTCTGctgTGTcGTcTcTTGCCTCTT| A
"ALPHA"
3' ---CTGCTTGTTCGCTCTGTGTC^ T
T-HAIRPIN

FIG. 20A

SEQUENCE OF ALPHA PRIMER:

5' GAC GAA CAA GCG AGA CAG CG 3'

FIG. 20B

CLEAVED A - HAIRPIN

CLEAVED T - HAIRPIN

FIG. 20C

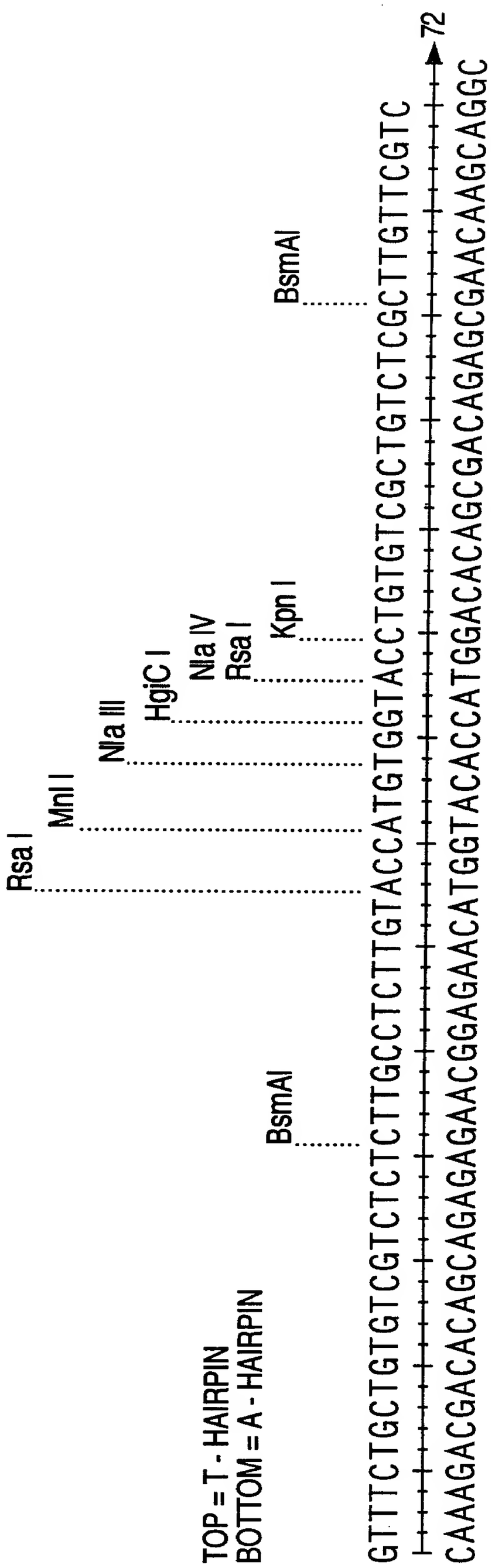

BsmAI
Rsa I
MnlI
Nla III
HgiC I
Nla IV
Rsa I
Kpn I
BsmAI
72
TOP = T - HAIRPIN
BOTTOM = A - HAIRPIN

FIG. 20D

FIGURE 21

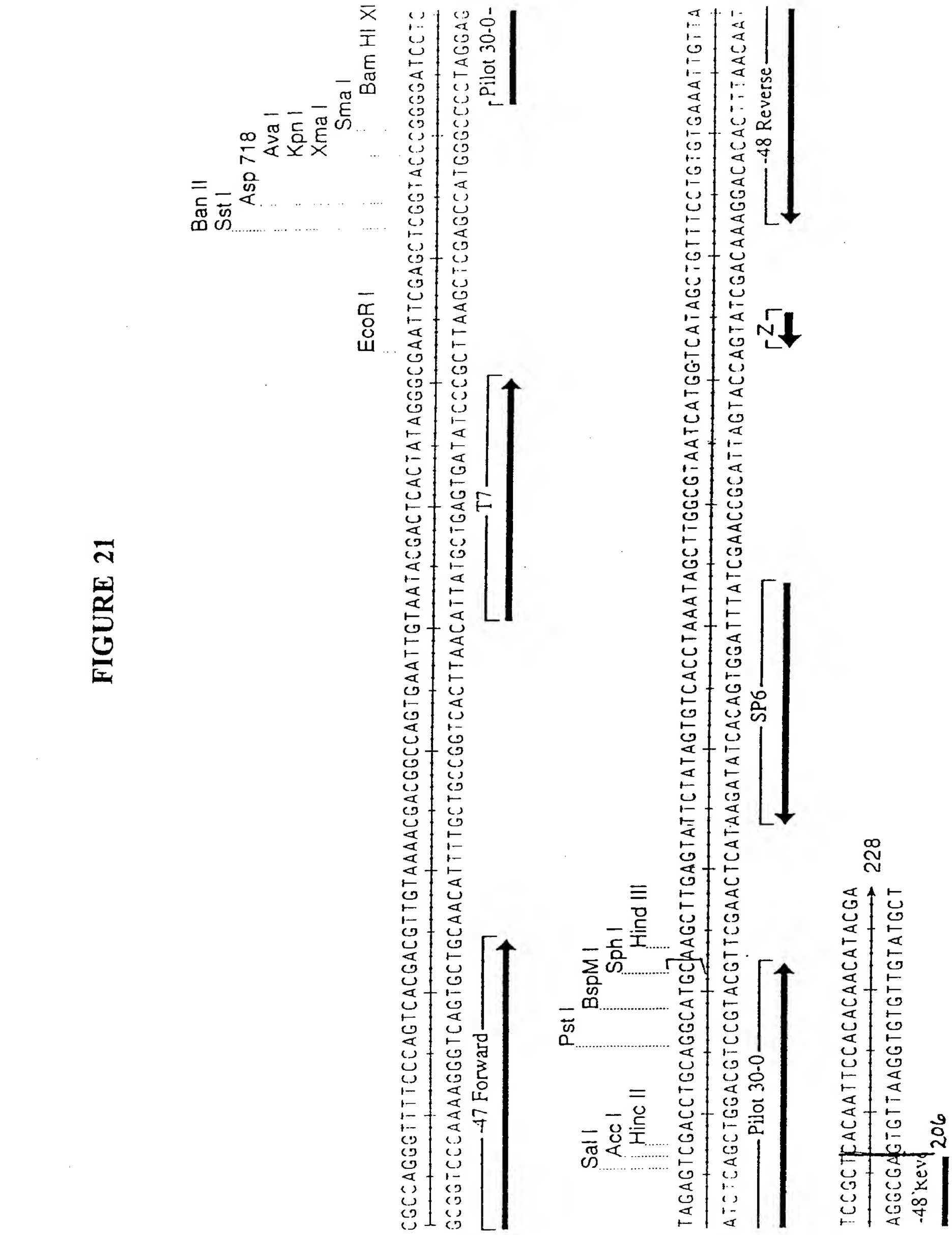
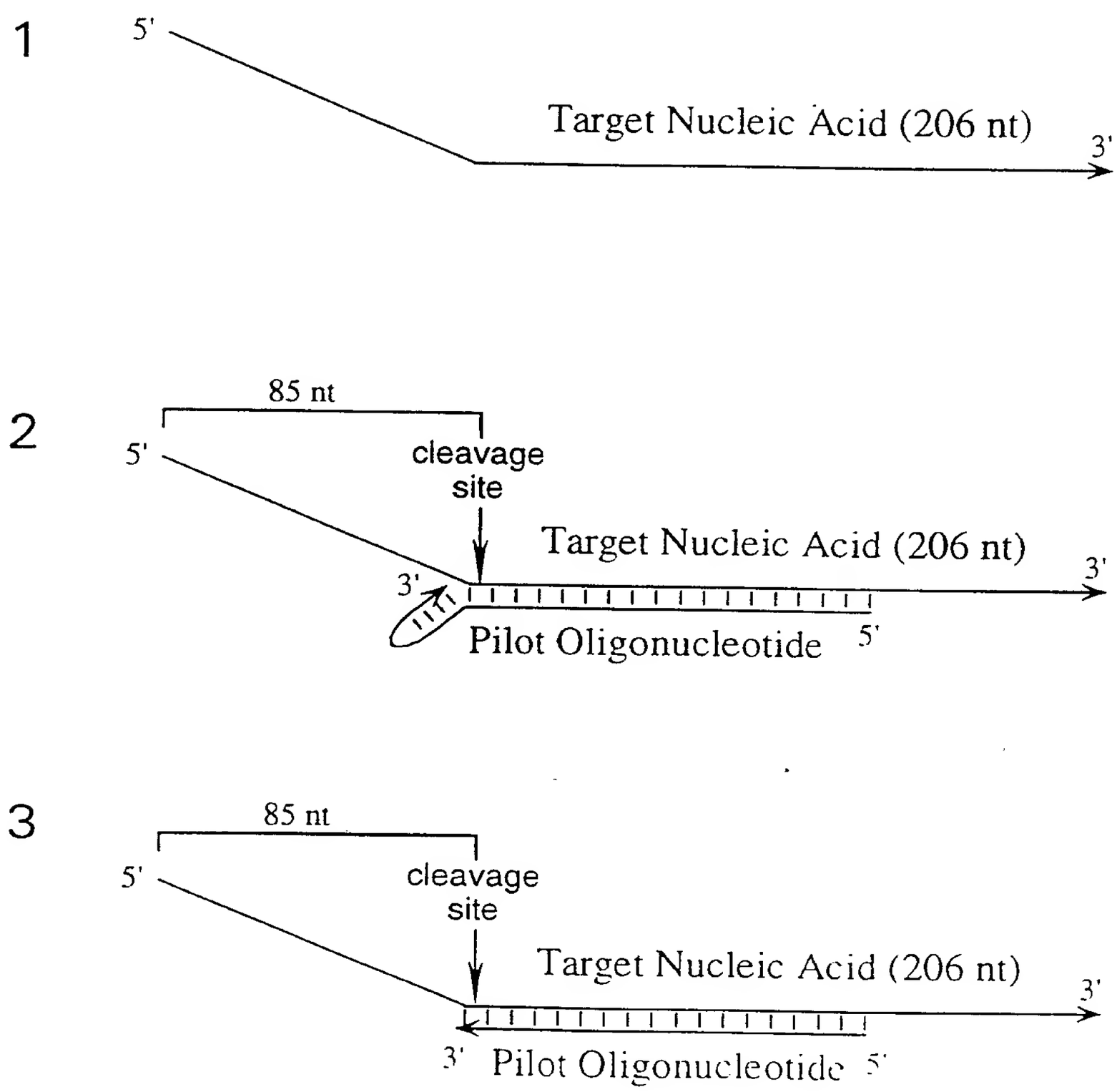

Ban II
Sst I
Asp 718
Ava I
Kpn I
Xma I
Sma I
Bam HI XI
EcoR I
Pilot 30-0-
T7
-48 Reverse
-47 Forward
SP6
Z
Pst I
BspM I
Sal I
Acc I
Hinc II
Sph I
Hind III
Pilot 30-0
228
206

FIGURE 22A



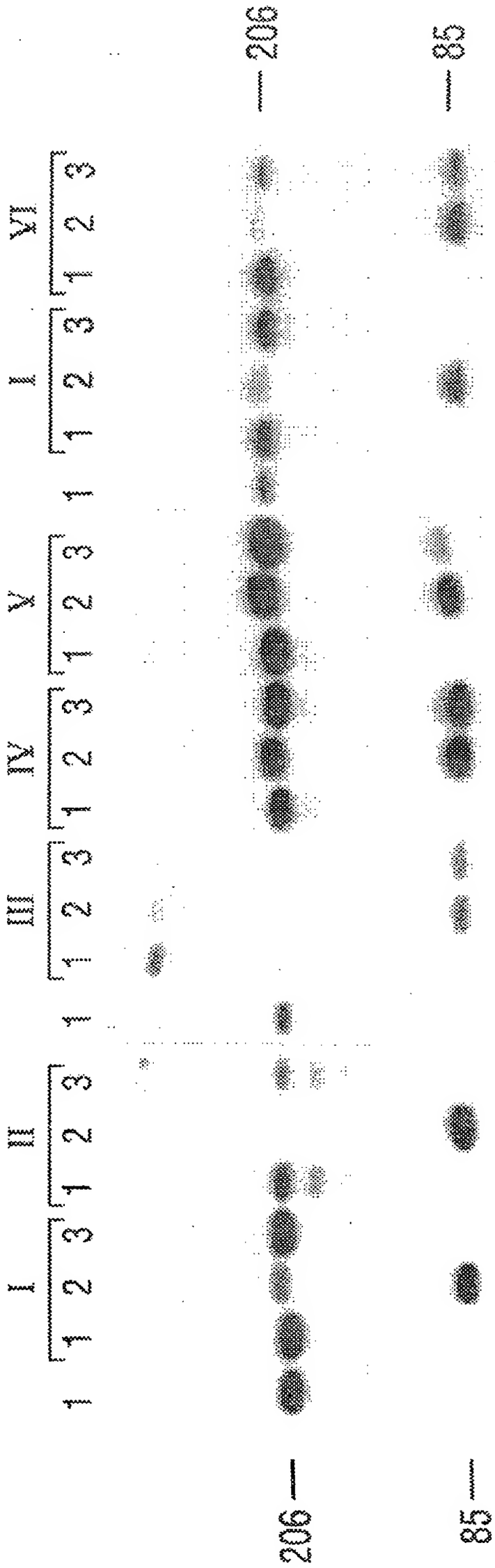
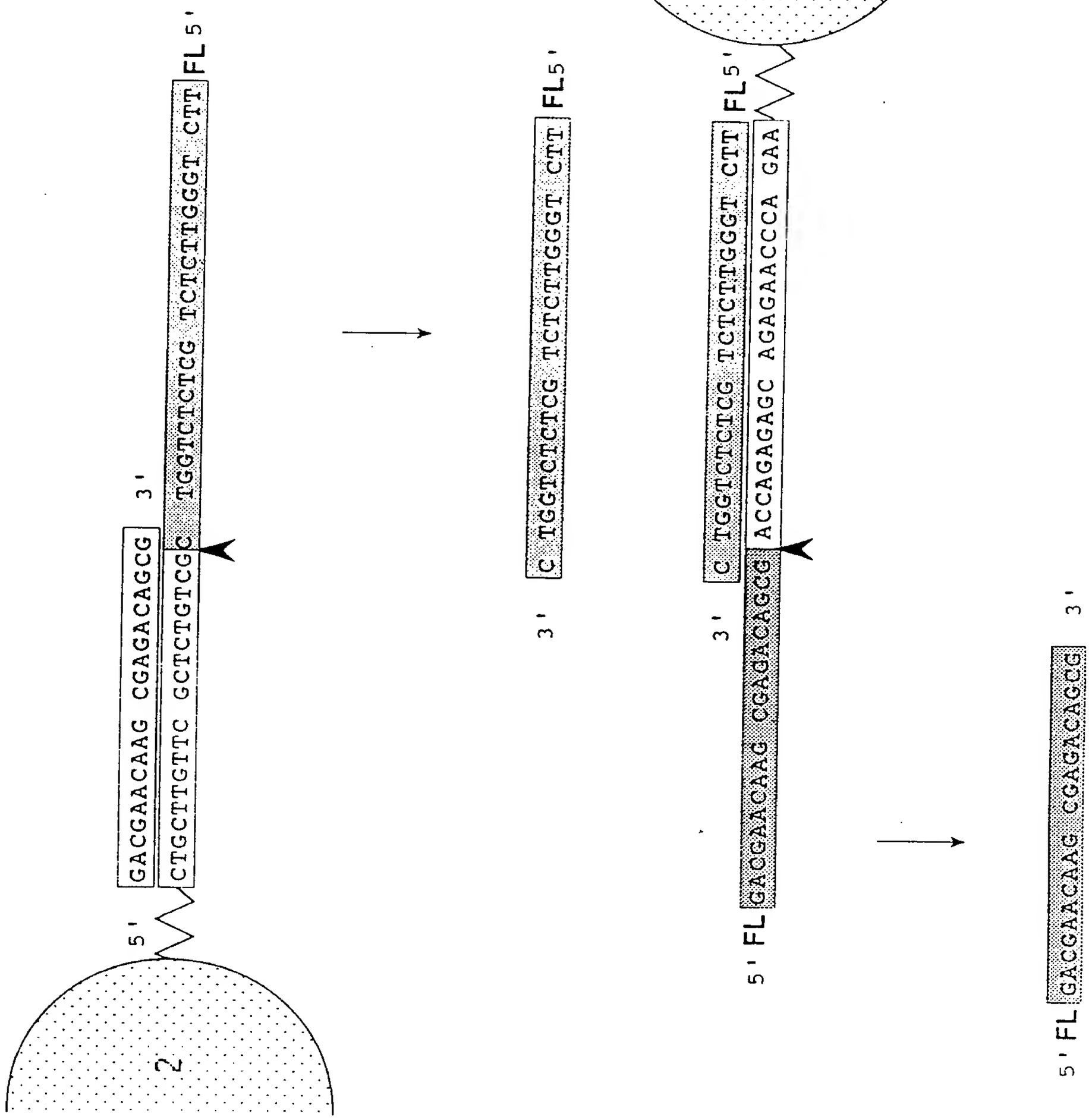


FIG. 22B

FIGURE 23


5' GACGAACAAG CGAGACAGCG 3'
CTGCTTGTTC GCTCTGTCGC TGGTCTCTCG TCTCTTGGGT CTT FL 5'
2
3' C TGGTCTCTCG TCTCTTGGGT CTT FL 5'
3
3' C TGGTCTCTCG TCTCTTGGGT CTT
5' FL GACGAACAAG CGAGACAGCG ACCAGAGAGC AGAGAACCCA GAA
5' FL GACGAACAAG CGAGACAGCG 3'

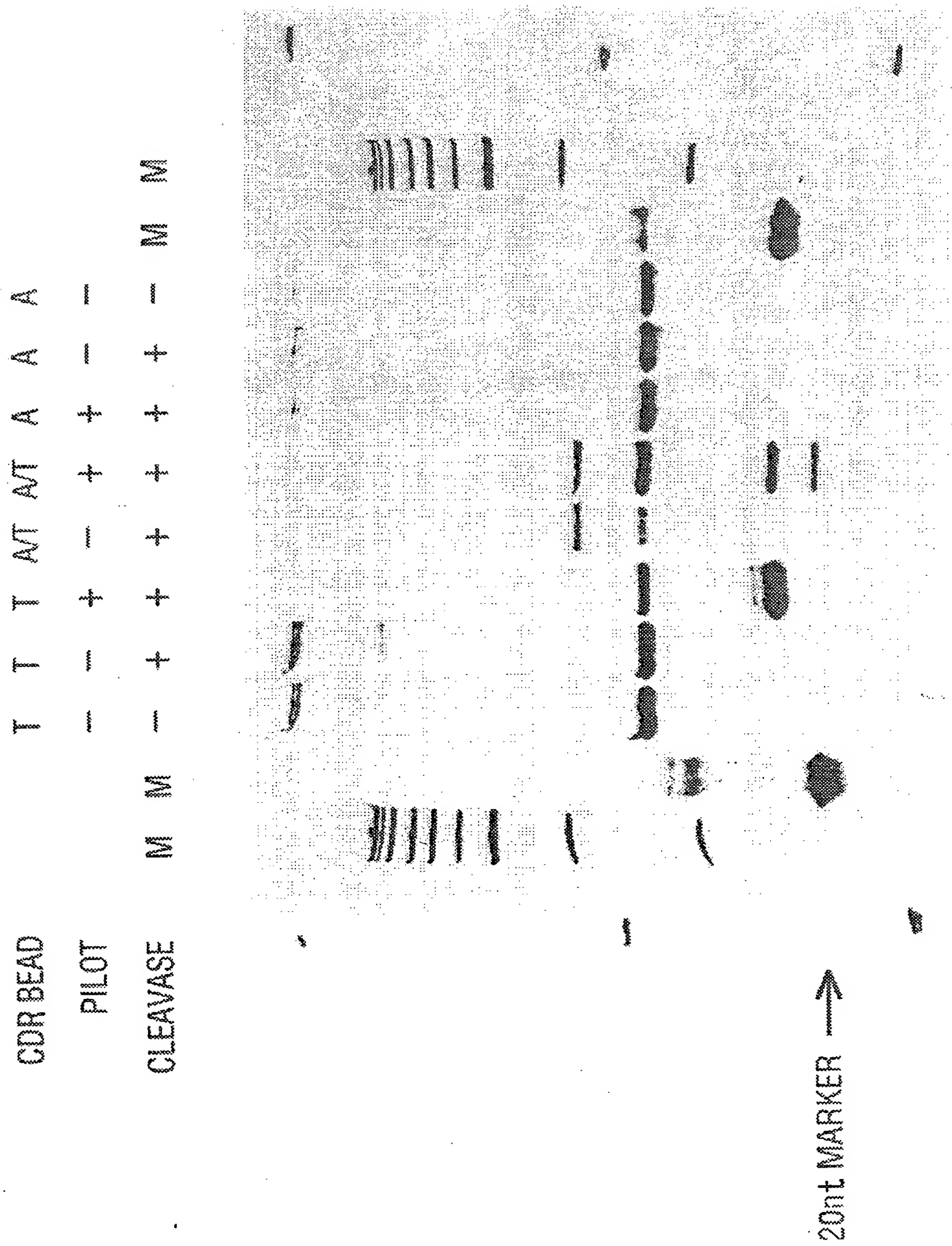

24nt MARKER
M M
- -
- +
+ -
+ +
T T
T T
AT AT
A A
A A
- -
+ -
- +
+ +
M M
20nt MARKER
COR BEAD
PILOT
CLEAVASE

FIG. 24

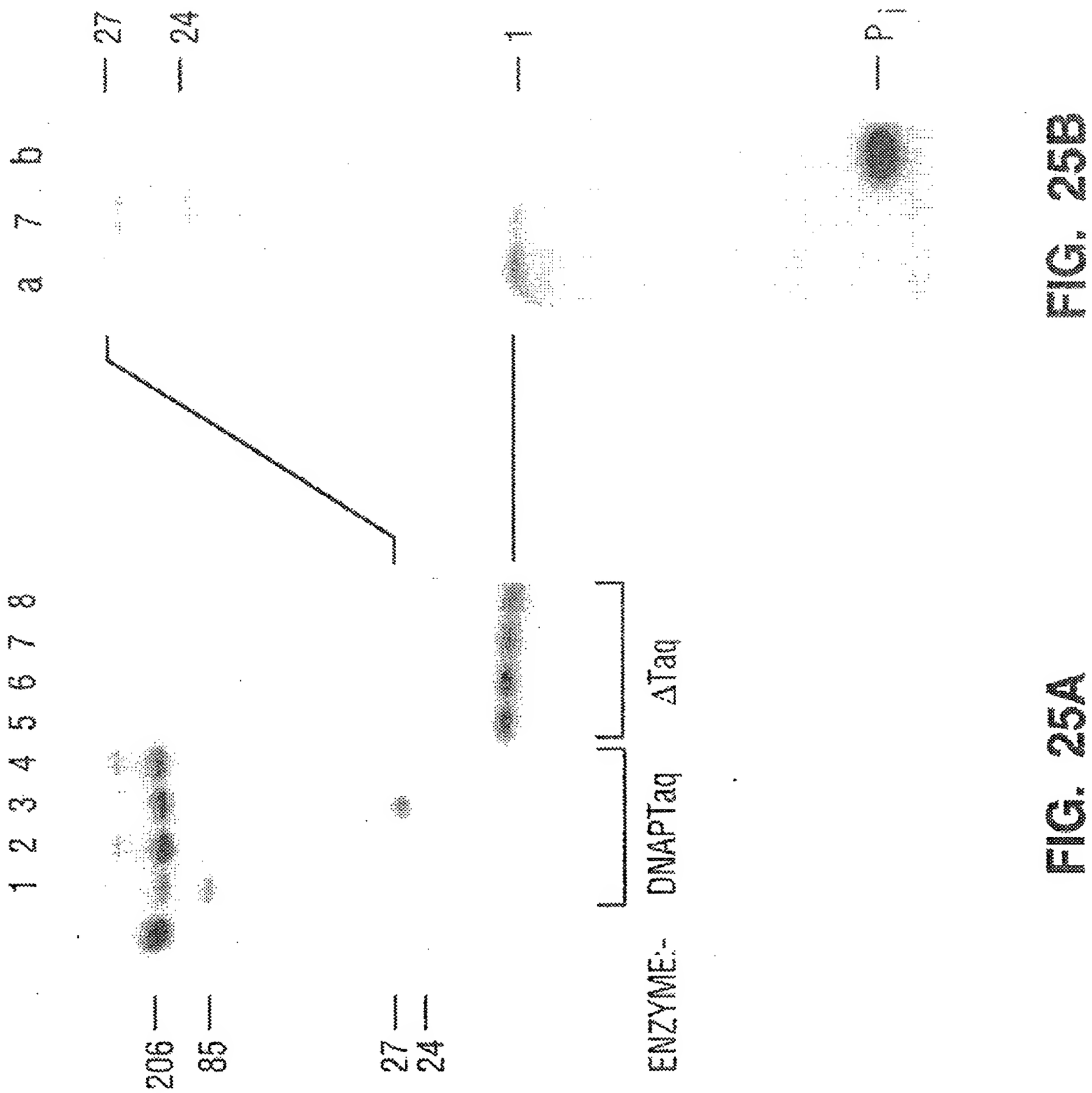


FIG. 25A

FIG. 25B

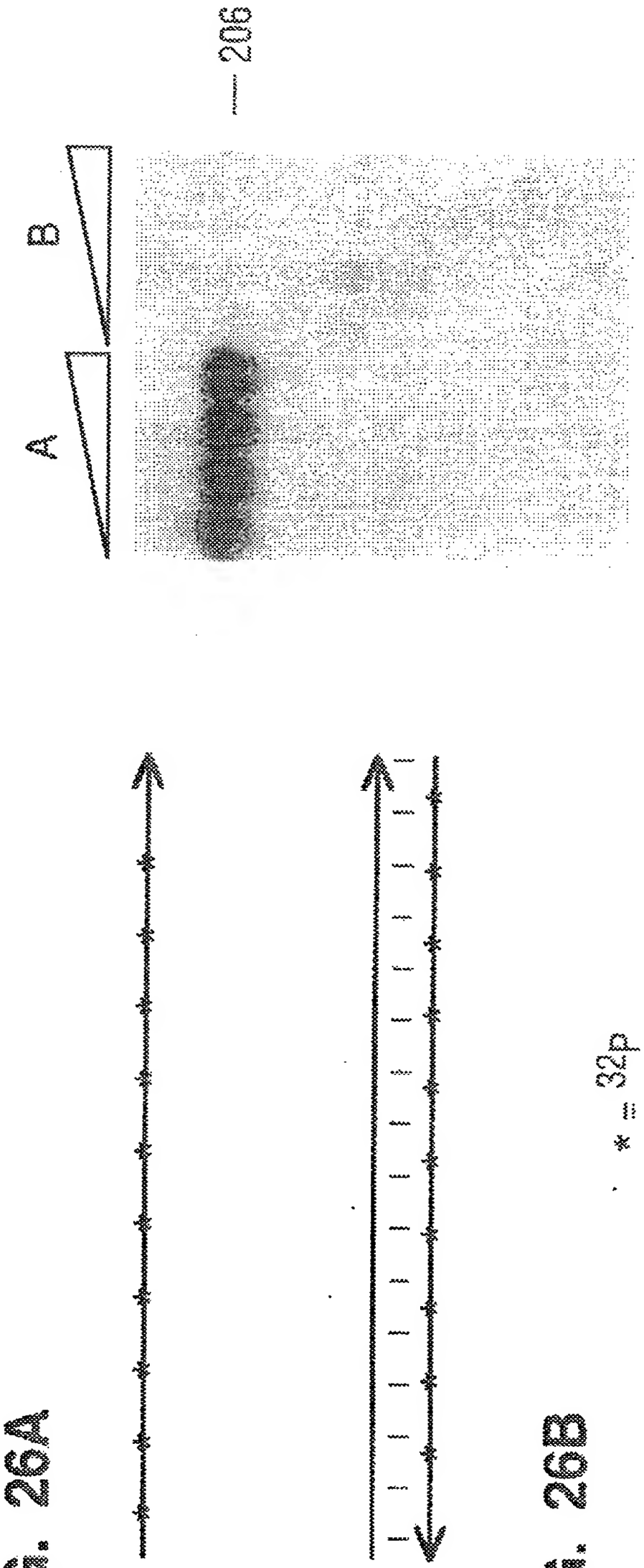
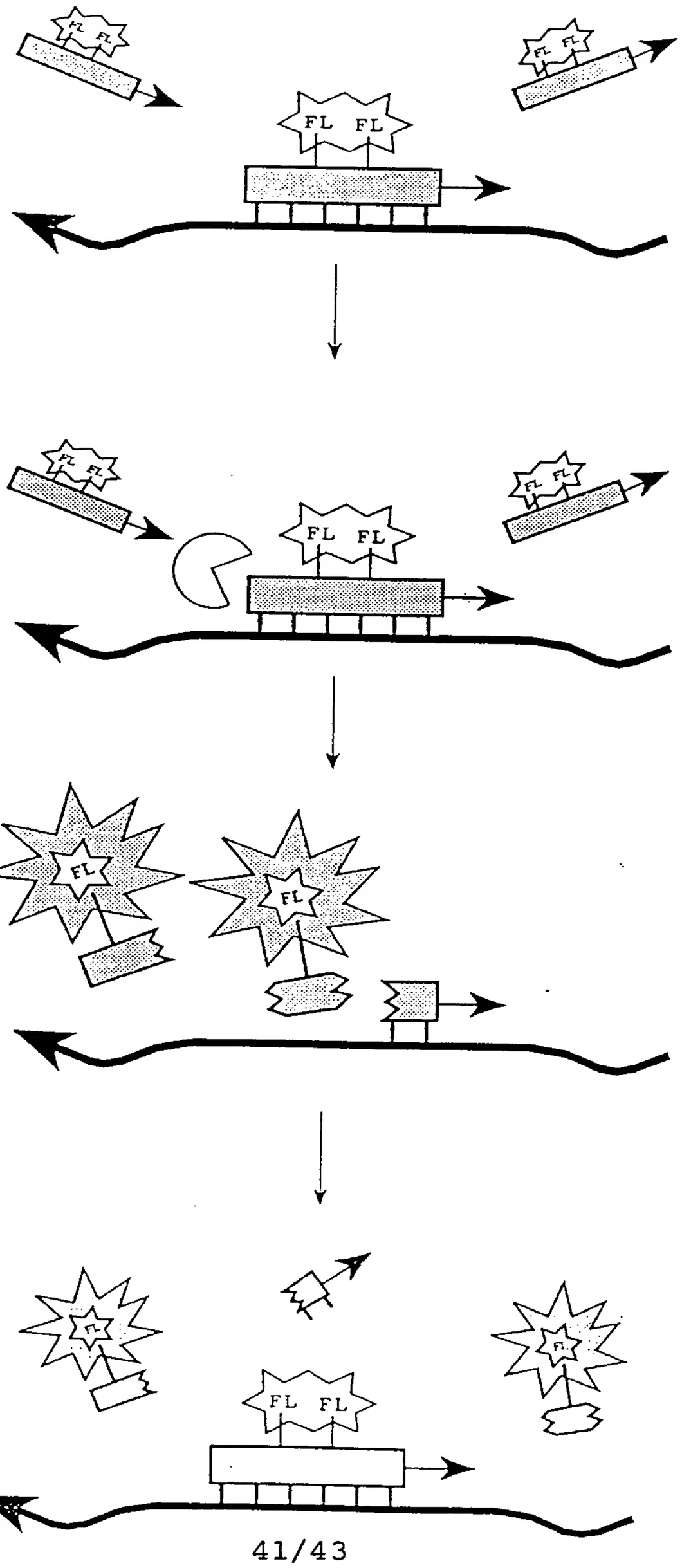


FIG. 26A

FIG. 26B

FIGURE 27



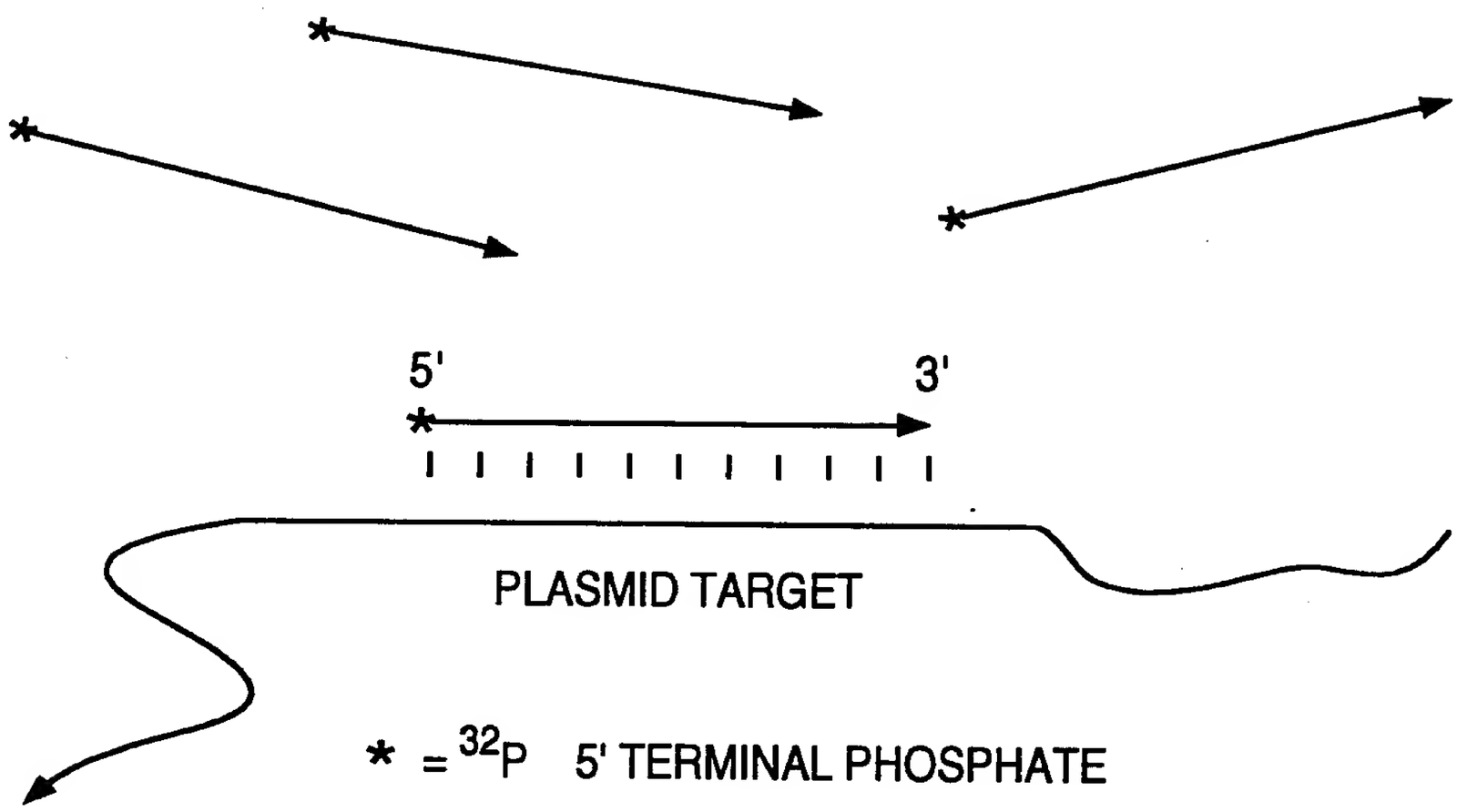


FIG. 28A

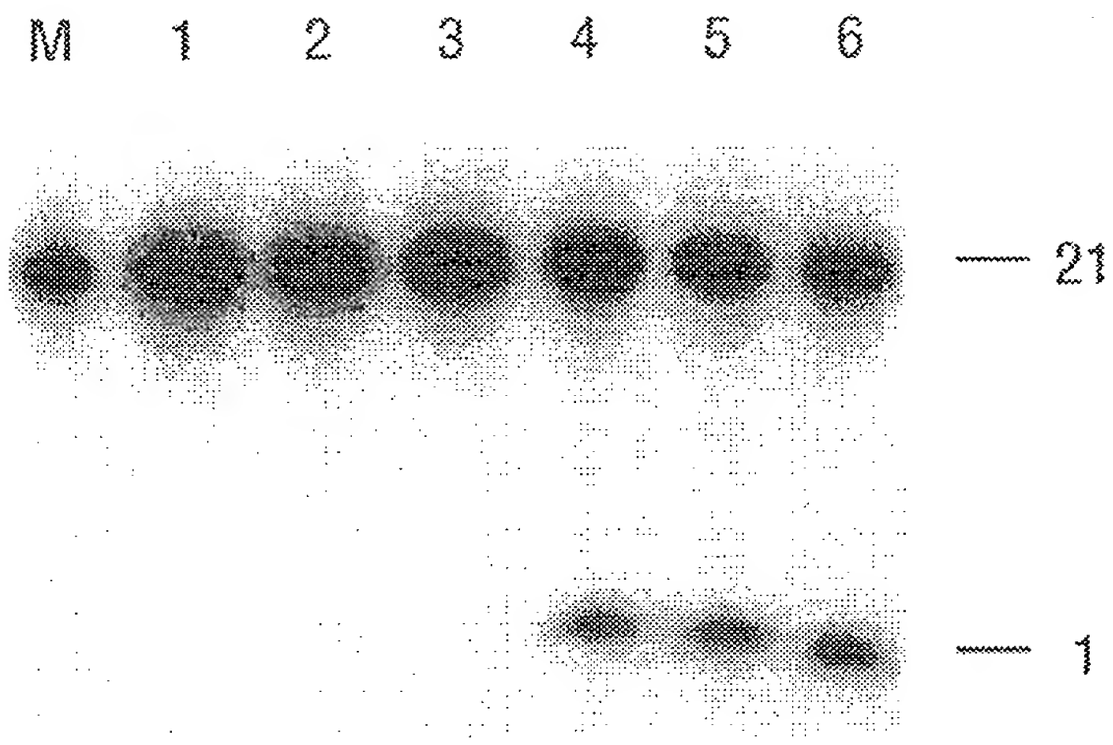


FIG. 28B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06253

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/6, 91.1, 172.3, 252.1, 320.1; 530/350; 536/23.7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 172.3, 252.1, 320.1; 530/350; 536/23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA
search terms: synthesis, polymerization, polymerase, deficient, thermostable, Taq, lack

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A, 482714 (SULLIVAN) 29 April 1992, Abstract.	1, 3
Y	Nucleic Acids Research, Volume 18, No. 24, issued 1990, M. J. Longley et al, "Characterization of the 5' to 3' exonuclease associated with Thermus aquaticus DNA polymerase", pages 7317-7322, especially page 7320, Substrate Specificity and page 7319, second column.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
25 AUGUST 1994

Date of mailing of the international search report
SEP 06 1994

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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06253

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 264, No. 11, issued 15 April 1989, F. C. Lawyer et al, "Isolation, Characterization, and Expression in Escherichia coli of the DNA Polymerase Gene from Thermus aquaticus", pages 6427-6437, especially Figures 2 and 7, Materials and Methods, and page 6434, second full paragraph.	1-19
Y	Proceedings of the National Academy of Science USA, Volume 88, issued August 1991, P. M. Holland et al, "Detection of specific polymerase chain reaction product by utilizing the 5'-->3' exonuclease activity of Thermus aquaticus DNA polymerase", pages 7276-7280, especially page 7276, paragraph bridging first and second column and Figure 1.	1-19
Y	Proceedings of the National Academy of Science USA, Volume 82, issued January 1985, T. A. Kunkel, "Rapid and efficient site-specific mutagenesis without phenotypic selection", pages 488-492, especially Abstract.	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Telephone Practice
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06253

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68; C12P 19/34; C12N 1/21, 9/16, 15/63, 15/70; C07H 21/02, 21/04

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-13, drawn to DNA encoding a thermostable polymerase altered in nucleotide sequence such that it exhibits altered DNA synthetic activity from that of the native enzyme, a vector carrying the DNA, and a host carrying the recombinant vector, classified in Class 536, subclass 23.7, for example.
II. Claims 14-25, drawn to a thermostable polymerase altered in nucleotide sequence such that it exhibits altered DNA synthetic activity from that of the native enzyme and a method of detecting the presence of a specific target DNA molecule utilizing complementary and partially complementary oligonucleotides which form hairpin cleavage structures and the altered thermostable polymerase, classified in Class 530, subclass 350, for example.
III.Claims 26-29, drawn to a method of detecting the presence of a specific target DNA molecule utilizing support bound partially complementary oligonucleotides which form cleavage structures which are not hairpin structures, classified in Class 435, subclass 6.